Gamma-aminobutyric acid-mediated neurotransmission in insects

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γ-AMINOBUTYRIC ACID - MEDIATED NEUROTRANSMISSION IN INSECTS

submitted by Timothy Neil Robinson

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SUMMARY

A centrifugation assay and a filtration assay were developed to measure the binding of [^{3}H]muscimol and [^{3}H]flunitrazepam, respectively, to a washed membrane preparation from the supraoesophageal ganglion of the locust, Schistocerca gregaria. The membranes exhibited high and low affinity, specific, saturable, Na^+-independent binding of [^{3}H]muscimol of K_D 10 and 80 nM, and B_{max} 70 and 400 fmol/mg protein respectively. The binding of [^{3}H]muscimol was insensitive to bicuculline methiodide. The membranes exhibited Ca^{2+}-dependent binding of [^{3}H]flunitrazepam of K_D 17-47 nM and B_{max} 500 $\pm$ 100 fmol/mg protein. Ro5-4864 was the most potent of a range of benzodiazepines in displacing [^{3}H]flunitrazepam binding, while up to 100% enhancement of binding was observed in the presence of GABA or isoguvacine. [^{3}H]Flunitrazepam photoaffinity labelled two major proteins of M_r 46K and 57K in locust ganglionic membranes. A single protein of M_r 48.5K was photolabelled in rat brain membranes in parallel experiments.

A homogenate was produced from locust supraoesophageal ganglion which was shown by electron microscopy to be rich in synaptosomal profiles. This homogenate accumulated 600 pmol [^{3}H]choline/mg protein following a 30 min incubation at 30°C. The uptake was resistant to metabolic perturbation, but was blocked by

incubation in the presence of detergent or hemicholinium-3, or on ice. Following preloading of the homogenate with [^{3}H]choline, radioactivity was released by elevated K^+ (50 mM), partly in the form of [^{3}H]acetylcholine. The [^{3}H]choline uptake capacity was used as a test of the physiological viability of the homogenate under different physical conditions. The synaptosome-rich homogenate also accumulated 8 pmol [^{3}H]GABA/mg protein following a 20 min incubation at 30^{o}C. This uptake was blocked by low temperature, elevated K^+ (50 mM), or 2,4-diaminobutyrate, but was unaffected by AOAA or gabaculline. A preliminary study was made of [^{3}H]GABA release from the homogenate.

A short study was made of putative GABA receptors on locust muscle. Membranes from the flight muscle exhibited specific binding of [^{3}H]flunitrazepam which was most potently displaced by Ro5-4864, but only low-affinity, non-saturating binding of [^{3}H]muscimol was observed to the same membranes. In an electrophysiological study, GABA-evoked responses were demonstrated in the extensor tibiae muscle, which were sensitive to muscimol, isoguvacine, bicuculline and picrotoxin. Diazepam had no consistent effect on the responses.

ABBREVIATIONS

ACh - acetylcholine

AOAA - aminooxyacetic acid

3-APS - 3-aminopropanesulphonic acid

AVM - avermectin B_{1a}

BZ - benzodiazepine

cAMP - cyclic adenosine monophosphate

CBR - central benzodiazepine receptor

CI - common inhibitor axon

Ch - choline

CNS - central nervous system

DABA - 2,4-diaminobutyric acid

DDT - 1,1'-(2,2,2-trichloroethylidene) bis[4-chlorobenzene]

DNP - 2,4-dinitrophenol

DPTXN - dihydropicrotoxin

DUMETi - dorsal unpaired median axon

EDTA - ethylene diamine tetracetic acid

EGTA - ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ET - extensor tibiae

FETi - fast axon

GABA - γ-aminobutyric acid

GABA-T - 4-aminobutyrate-2-oxoglutarate transaminase

GAD - l-glutamate l-carboxy-lyase

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GC-MS - gas chromatography-mass spectroscopy

H - homogenate

HC-3 - hemicholinium-3

HEPES - N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid

HF - filtered homogenate

IPSP - inhibitory postsynaptic potential

LDH - lactate dehydrogenase

MDH - malate dehydrogenase

mipps - miniature inhibitory postsynaptic potentials

mRNA - messenger ribose nucleic acid

NADH - nicotinamide adenine dinucleotide

NADPH - nicotinamide adenine dinucleotide phosphate

NMJ - neuromuscular junction

PAGE - polyacrylamide gel electrophoresis

PBR - peripheral benzodiazepine receptor

P_2G - locust ganglionic membrane preparation

PLP - pyridoxal phosphate

P_2M - locust flight muscle membrane preparation

PMSF - phenylmethylsulphonylfluoride

PPO - 2,5-diphenyloxazole

P_2R - rat brain membrane preparation

PTXN - picrotoxin

SDS - sodium dodecyl sulphate

SETi - slow axon

SSA - succinate semialdehyde

SSADH - succinate semialdehyde dehydrogenase

TBPS - t-butylbicyclophosphorothionate

tlc - thin layer chromatography

Tris - 2-amino-2-(hydroxymethyl)propane-1,3-diol

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CHAPTER 1

GENERAL INTRODUCTION

1.1/ THE DEVELOPMENT OF OUR UNDERSTANDING OF NERVOUS SYSTEMS

In the nineteenth century it was generally accepted that the nervous system was not composed of discrete cells, but rather that it was one single network. However, based on histological observations, Ramon y Cajal (1888) proposed that the nervous system was in fact composed of discrete units, the nerve cells or neurons, which communicated with each other via specialised contacts. This theory was extended by the electrophysiologist, Sherrington (1925), who demonstrated that the nervous system was electrically discontinuous and called the specialised contacts, observed by Ramon y Cajal, "synapses". This electrical discontinuity of the nervous system suggested that some additional form of communication must be located at the synapse, and Elliot (1904) and Dixon (1906) postulated that a chemical messenger is released at the synapse which transmits information to surrounding cells. Loewi (1921) reported a set of experiments in which he chronically stimulated the vagus nerve of an isolated frog heart and then observed that the resulting bathing medium mimicked vagal stimulation when applied to a second heart. This confirmed that a chemical could indeed initiate nervous activity and formed the basis of the neurotransmitter theory of synaptic

neurotransmission. Subsequent to these pioneering experiments it has been recognised that there are several criteria that must be fulfilled by a chemical if it is to be classed as a neurotransmitter (Werman, 1966) and these are listed below:

1) The chemical is synthesised and stored in the nerve terminal.

2) The chemical is released on stimulation of the nerve.

3) The chemical produces a change in polarization by an interaction at the post-synaptic site.

4) The quantity of chemical so released is sufficient to initiate either depolarization or hyperpolarization.

5) There exists a method of removal from the synapse for the chemical.

By the early 1970s it was widely believed that most of the important neurotransmitters utilized by the mammalian brain had been discovered, though not all the above criteria were necessarily fulfilled in every case. The suggestion that a chemical was a neurotransmitter usually followed the observation of some salient feature of the substances' physiology or pharmacology. For example, substance P was originally identified in tissue extracts by its effects on smooth muscle motility and non-cholinergic pharmacology (see von Euler, 1985). It was considered that the CNS could function effectively with the dozen or so known excitatory or inhibitory substances. However, more recently there has been an

explosion in the number of putative neurotransmitters in the mammalian brain. This is largely due to the discovery of neuroactive peptides, such that in 1979 some 30 different substances were thought to be transmitters in the brain (Iversen, 1979). By 1983 the list of neuroactive peptides alone numbered 37 (Krieger, 1983) and today there are thought to be in excess of 50 putative neurotransmitters in the mammalian CNS and the number is still increasing (Rossier, 1985). This makes a highly complex picture of the vertebrate brain with many questions regarding the interactions and control of all these different agents still to be answered. But what is our knowledge of the insect nervous system and its neurotransmitters?

1.2/ THE INSECT CENTRAL NERVOUS SYSTEM

The anatomical organization of the insect nervous system has been well characterized and a detailed description is given by Chapman (1975). Briefly, it consists of a brain situated dorsally in the head and a ventral chain of segmental ganglia from which nerves run to the peripheral sense organs and to the muscle systems. A stomatogastric system, consisting of a number of small ganglia connected to the brain and their associated nerves , controls the movements of the alimentary canal.

The supraoesophageal ganglion of the locust

represents the brain and is the principal association centre of the animal's body. It is also the seat of many long-term organized behavioural patterns and governs their modification by learning. It is this locust brain which was used throughout the work on insect central nervous tissue described in this thesis.

Our knowledge of the structural organization of the insect nervous system and its relative simplicity have often made insects the animals of choice for electrophysiological studies. In contrast with the mammals though, we know much less about the chemical events involved in invertebrate neurotransmission. This ignorance represents a great imbalance in our knowledge when one considers that there are over 800,000 species comprising the phylum Arthropoda, of which species of insects are by far the major constituents, whereas 47,000 different species comprise the phylum Chordata which includes the whole spectrum of vertebrates. The number of postulated neurotransmitter candidates in the insect nervous system (Table 1) is very limited compared with the situation described above for mammalian brain, and this limited knowledge is based largely on electrophysiological studies. This lack of knowledge is one aspect of the general problem posed by the very small quantities of nervous tissue available to the invertebrate neurochemist. Indeed we have only seen any real progress in the case of acetylcholine, which is in part due to the fact that the insect CNS has a higher content of acetylcholine and a greater density of receptors for the transmitter than

Neurotransmitter	Site of Action
Acetylcholine[1]	Central nervous system
GABA[2]	Central nervous system
Glycine[2]	Central nervous system
L-Glutamate[2]	Neuromuscular junctions
GABA[2]	Neuromuscular junctions
L-Aspartate[3]	Neuromuscular junctions
5-Hydroxytryptamine[4]	Visceral & cardiac NMJ
Dopamine[4]	Visceral & cardiac NMJ
Octopamine[4]	Visceral & cardiac NMJ
Proctolin[4]	Visceral & cardiac NMJ

1 - Sattelle, 1980.

2 - Usherwood, 1978.

3 - Irving & Miller, 1980.

4 - Miller, 1980.

Table 1 Putative neurotransmitters in insects.

mammalian brain (Breer, 1981b). However in the last ten years there has been an increased effort and interest in the field of invertebrate neurochemistry, not only from an academic standpoint (see The Report of the Invertebrate Neuroscience Panel of the U.K. Science & Engineering Research Council, 1986) but also due to increased interest on the part of the pesticide industry.

1.3) A BRIEF HISTORY OF THE PESTICIDE INDUSTRY

In the early 1930s the only pesticides in use were highly toxic compounds such as lead arsenate (and insects such as cattle ticks even developed resistance to arsenate) and there were only a few neuropoisons, such as nicotine, available. Such compounds were of little use in the major fields of pest control, namely agriculture and public health.

With the onset of World War II so there was increased research into neuropoisons and an offshoot of this was the dicovery of highly active pesticidal agents such as the organochlorines (DDT for example) and the organophosphates. This marked the beginning of a new and booming era for the pesticide business such that in 1982 world sales of pesticides were in excess of £7 billion (Matthews, 1983).

However, two major problems rapidly reared their heads. Firsty the lack of specificity of pesticides, an example of which occurred in the Gezira region of the Sudan

where the cotton bollworm was an important pest. In response to this problem, broad-spectrum insecticides were applied which unfortunately had the dual effect of eradicating the natural predators of another enemy of cotton, namely cotton whitefly with the result that the flies proliferated and downgraded the cotton lint (Matthews, 1983). A second problem is that of resistance of pests to pesticides. This is an ever-increasing problem; in 1947 there were 14 pesticide-resistant species reported, but by 1976 the number had swollen to 364.

The pesticide industry is therefore constantly searching for new and more specific agents. As a result, in addition to the random screening of chemicals for pesticidal activity, the industry has also embarked on an increased activity in basic research with a view to identifying new targets for pesticides. Though most insecticides act by disruption of the nervous system of the insect, we are ignorant of the mode of action of many of today's insecticides. Some are known to act on cholinegic neurotransmission (organophosphates act on acetylcholine esterase but may have other, as yet undefined, targets) but this limited knowledge and our ignorance in the majority of cases is a reflection of our knowlege of insect nervous systems as a whole, namely that we only know about cholinergic neurotransmission in any depth. Therefore the pesticide industry needs increased

basic research into other invertebrate neurotransmission systems; GABA is the neurotransmitter of one such system.

1.4/ GABA AS A NEUROTRANSMITTER

γ-Aminobutyric acid (GABA) was discovered at the beginning of the century (Ackerman & Kutscher, 1910) and shortly after it was first detected in mammalian brain in 1950 (Awapara et al., 1950, Roberts & Frankel, 1950, Udenfriend, 1950), an extract of mammalian brain and spinal cord was shown to contain a factor, 'Factor I', which inhibited the stretch receptor neuron (a single sensory cell with its dendrites entwined in a fine muscle bundle) of the crayfish (Florey, 1954). Fractional crystallization of bovine brain Factor I showed GABA to be its most active component in terms of inhibitory activity on the stretch receptor neuron (Bazemore et al., 1957). Following some controversy over GABA's role as a neurotransmitter, Kravitz and colleagues demonstrated GABA to be present in lobster inhibitory neurons where it is synthesized, accumulated and released (Kravitz, 1967), and this work is described later in this chapter (1.5). By the early 1970s it was thought that GABA may be the universal transmitter of junctional neuromuscular inhibition in all invertebrate phyla from nematodes to arthropods (though not molluscs) and it was also thought to be involved in the CNS of

crustaceans, insects and possibly molluscs (see Gerschenfeld, 1973; Pichon, 1974; Callec, 1974). However the evidence for this was virtually all electrophysiological and despite the early studies on GABA involving invertebrate tissues, the bulk of the biochemistry of GABAergic neurotransmission has been elucidated in mammalian tissues. There have been several detailed reviews of the now well-characterized mammalian GABA system (eg. Bradford, 1986) and it is depicted in a highly simplified form in Fig.1.

Essentially GABA is synthesized in the nerve terminal from glutamate by glutamate decarboxylase (GAD) and is released into the snynaptic cleft in response to stimulation of tne neuron. Binding of GABA to receptor complexes in the post-synaptic membrane causes the opening of associated chloride ion channels and hence hyperpolarization of the post-synaptic cell. Following dissociation of GABA from its receptor it is cleared from the synaptic cleft by diffusion and uptake by specific carriers into both neurons and glial cells. GABA can then be metabolized by GABA transaminase (GABA-T) to succinic semialdehyde which in turn is converted to succinate by succinate semialdehyde dehydrogenase (SSADH).

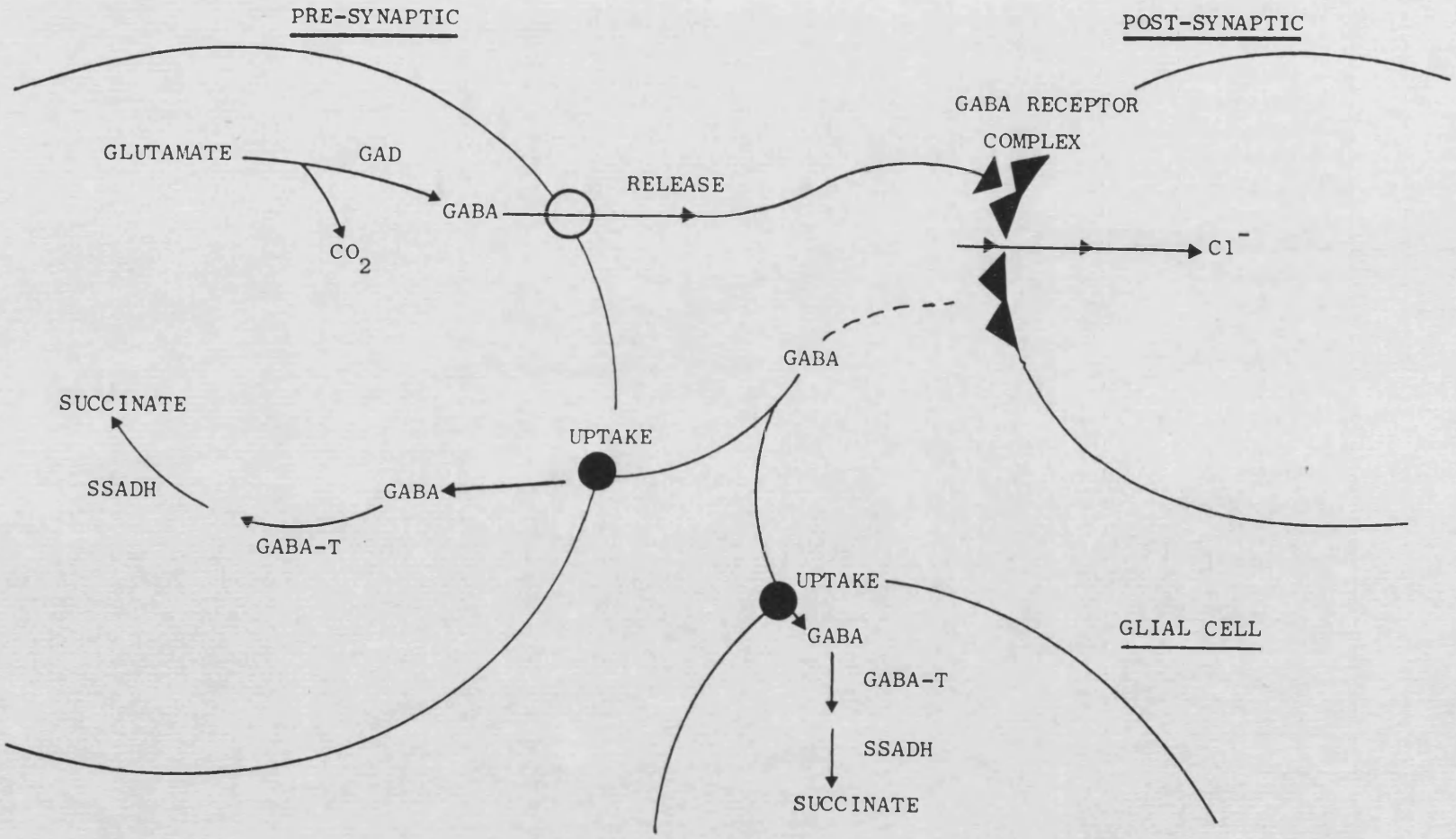


Fig.I Schematic representation of GABAergic neurotransmission in mammalian CNS.

1.5/ GABA AS AN INVERTEBRATE NEUROTRANSMITTER

Apart from the early work on the synthesis and metabolism of GABA by Kravitz and colleagues, investigations of an invertebrate GABAergic system comparable with that illustrated in Fig.1 have, until recently, been sparse. However the work that has been done is reviewed below.

1.5.1/ The GABA content of nervous tissues

Invertebrate nervous tissues have beem shown to possess GABA in concentrations which are comparable with, and in some cases higher than, those measured in mammalian nervous tissues. However the methods of measuring GABA in tissue extracts have changed over the years from using two-dimensional chromatography and ninhydrin treatment of the resulting spots (Ray, 1964), through detection of DNP-amino acid methyl esters by gas liquid chromatography (Aprison et al., 1973), detection of GABA by gas chromatography-mass spectroscopy (GC-MS) (McAdoo & Coggeshall, 1976), and more recently the microchemical procedure of Witte & Matthaei (1980) whereby GABA is assayed by digestion with GABAse (a mixture of GABA-T and SSADH) and the resulting $NADPH^+$ is measured. These variations in methodology should be borne in mind when comparing data for GABA concentrations in different tissues.

GABA was first shown by Kravitz et al. (1962)

to be present in crab peripheral nerves (10-12 μg/100 mg dry weight) and lobster ganglia (60 μg/100 mg dry weight). These workers then went on to demonstrate that in lobster inhibitory axons the concentration of GABA (100 μM) is 100-fold greater than that of glutamate (1 μM), while the GABA concentration of excitatory axons is only 0.6 μM, in accordance with GABA's postulated role as an inhibitory transmitter.

The situation in the molluscs is less clear since significant levels of GABA could not be detected in octopus brain (Roberts, 1964) or in the nervous system of the cephalopod, Sepula esculenta (Tsukada et al., 1964). Also in snail and octopus nervous tissue the incorporation of label from [U-^{14}C]glutamate could not be detected in GABA (Cory & Rose, 1969; Bradford et al., 1969). However subsequent to that Osborne et al. (1972) demonstrated small amounts of GABA in Helix ganglia.

In the annelids endogenous GABA levels were reported to be too low for detection by GC-MS (McAdoo & Coggeshall, 1976), though more recently Sargent (1977) has demonstrated that leech ganglia incubated with labelled glutamate do accumulate labelled GABA.

Insect (honeybee) brain was shown by Frontali (1964) to possess GABA in 2-3-fold higher concentrations than mammalian brain, and Baxter & Torralba (1975) have measured the concentration of GABA in the metathoracic ganglia (12.8 μmol/g dry weight) and abdominal ganglia (11.2 μmol/g dry weight) of the cockroach. Further data

for the GABA content of insect and crustacean nervous tissue are compared with parallel data for mammalian tissues in Table 2. Clearly there is a lot of evidence, in arthropods at least, that GABA is present in nervous tissues in significant quantities, comparable with mammalian nervous tissues, concordant with its role as a neurotransmitter.

1.5.2/ Synthesis and metabolism of GABA

In the 1960s Kravitz and colleagues, using extracts from the lobster, found that the metabolic pathway for the synthesis and metabolism of GABA in the crustacean nervous system was very similar to that in the mammalian nervous system (Kravitz, 1967). GABA is produced by the decarboxylation of glutamate, with the concomitant production of CO_2, by the enzyme GAD (L-glutamate 1-carboxy-lyase, EC 4.1.1.15).

Metabolism of GABA is achieved by its transamination in the presence of 2-oxoglutarate to give succinic semialdehyde (SSA) and glutamate, by the enzyme GABA-T (4-aminobutyrate-2-oxoglutarate transaminase, EC 2.6.1.19). The resulting SSA is then oxidised in the presence of NAD^+ by SSA dehydrogenase to give succinate.

This metabolic pathway of GABA in nervous tissues is summarized in Fig.2. Both GAD and GABA-T have been purified from various vertebrate sources. However in the invertebrates, since the early studies in

Table 2 Concentrations of GABA in the nervous tissues of different species. The data for GABA concentration are expressed as μmol/g wet weight. The different methods employed for the measurement of GABA concentration are summarized below:
Frontali, (1964) - Spinco amino acid analyser.
Ray, (1964); Tallan, (1962) - Ninhydrin treatment of two-dimensional chromatograms.
Aprison et al., (1973)- Measurement of DNP-amino acid esters by GLC.
Breer & Heilgenberg, (1985)- GABAse digestion and measurement of $NADPH^{+}$.

Species	Tissue	GABA Conc.	Reference
Honeybee	Brain	10.9	Frontali, 1964
Cockroach	Thoracic & abdominal ganglia	2.5	Ray, 1964
Rat	Brain	2-6.1	Tallan, 1962
Rabbit	Brain	2.7	
Hen	Brain	2.7-6	
Lobster	Ganglia	0.95-3.6	Aprison et al., 1973
	Axonal connectives	0.91-2.6	
Locust	Cerebral ganglia	21.5	Breer & Heilgenberg, 1985
	Optic lobes	13.1	
	Thoracic ganglia	10.7	
	Abdominal ganglia	12.1	
Fish	Telencephalon	3.2	"
	Tectum opticum	3.8	
	Cerebellum	2.4	
Mouse	Cortex	2.4	"
	Cerebellum	2.2	

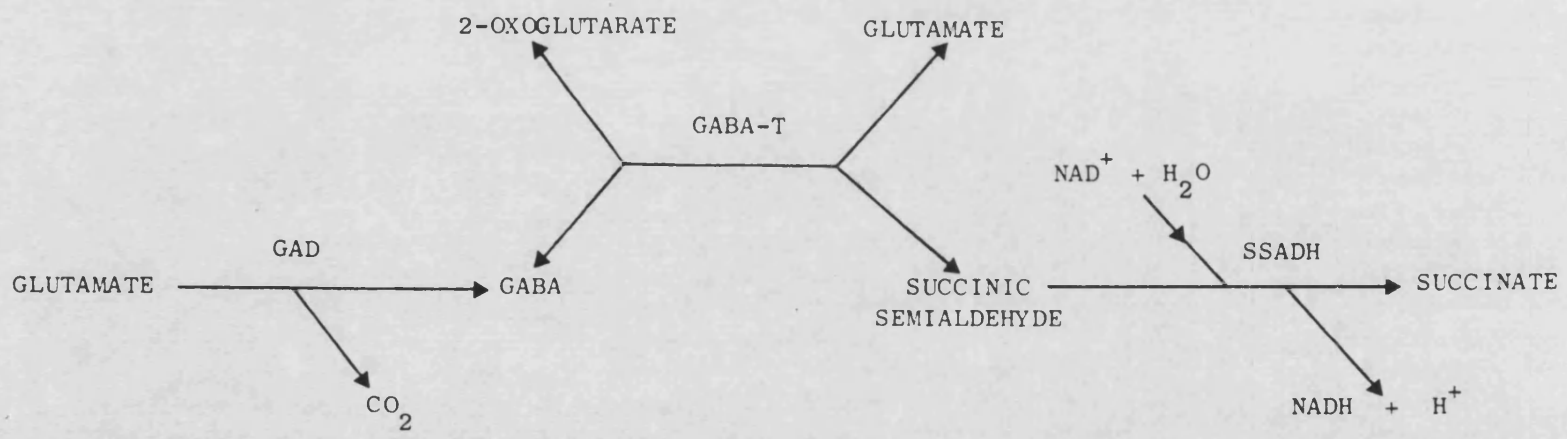


Fig.2 Metabolic pathway of GABA in mammalian nervous tissue.

lobster, there has only been a limited amount of work on GABA-T and marginally more on GAD, largely in insect tissues. Recently though both enzymes have been purified from insect nervous tissue.

1.5.2.1) THE SYNTHESIS OF GABA

Frontali (1961) detected the presence of GAD in insect nervous tissue and demonstrated that this enzyme required pyridoxal phosphate (PLP, 4×10^{-4} - 10^{-3} M) for optimal activity. Furthermore the insect GAD exhibited two pH optima of 7.2 and 8.0. Glutamate has also been shown to be converted to GABA in the CNS of locust (Bradford et al., 1969; Sugden & Newsholme, 1977), the moth, Manduca sexta (Maxwell et al., 1978) and cockroach thoracic ganglion (Huggins et al., 1967). Working with extracts of lobster, Molinoff & Kravitz (1968) obtained GAD activity with a single pH optimum of 8.0; potassium ions and 2-mercaptoethanol are essential for the enzyme's activity and it is competitively inhibited by GABA, implying a possible role for GAD in regulating GABA levels. Furthermore, GAD activity was found to be 100-fold higher in inhibitory than in excitatory lobster axons (Kravitz et al., 1965). Glutamate has also been shown to be converted to GABA in the neuronal somata of the lobster (Otsuka et al., 1967; Potter, 1968) and the ventral nerve photoreceptors of the horseshoe crab (Battelle et al., 1979). The enzyme has been purified from a number of mammalian sources. Wu

et al. (1973) reported the mouse brain enzyme to have an M_r of 85k, whereas human brain GAD has an M_r of 140k (Blinderman et al., 1978). Both enzymes are thought to be dimeric. In contrast, the catfish brain enzyme, while having an M_r of 90k, may be tetrameric (Su et al., 1979). A number of studies have been made of GAD in crude extracts of invertebrate nervous tissue, including Drosophila larval brain (Chen & Widner, 1969), honeybee brain (Fox & Larsen, 1972), cockroach metathoracic ganglion (Baxter & Torralba, 1975) and crayfish brain (Wu et al., 1976). The enzyme has been partially purified from housefly (Chude et al.,1979), and very recently GAD has been purified 700-fold from locust brain by a Fast Protein Liquid Chromatography method (Stapleton, 1986). The native enzyme has an M_r of 97k as estimated by gradient PAGE. Under denaturing electrophoretic conditions locust brain GAD consisted of two subunits of M_r 44k and 51k and therefore appears to be dimeric as described for the vertebrate enzyme. The two-dimensional tryptic mapping of these subunits showed a considerable degree of homology between the respective separated peptides. Furthermore this enzyme has been used to raise antibodies for mapping invertebrate neurones for GAD activity (Stapleton, 1986). Until purified by Stapleton (1986), invertebrate GAD consistently exhibited a lower affinity for glutamate than purified vertebrate GAD . Such differences in K_M may be a function of the different purities of the preparations, such that crude

preparations contain enzymes other than GAD which compete for glutamate, though the affinity of GAD for glutamate was decreased rather than increased on partial purification from mouse brain (Susz et al., 1966). However, though both insect and crustacean GAD have, if anything, a lower affinity for glutamate than the mammalian enzyme, insect nervous tissues have a higher activity of GAD (~10-fold) than either crustacean or vertebrate CNS (Baxter & Torralba, 1975).

A further difference between insect, crustacean and vertebrate GAD is the effects of potassium and chloride ions on GAD activity. Both insect GAD (Baxter & Torralba, 1975; Breer & Heilgenberg, 1986) and mammalian GAD (Susz et al., 1966; Blinderman et al., 1978) are inhibited by chloride ions whereas the lobster enzyme is not (Molinoff & Kravitz, 1968). Baxter & Torralba (1975) have suggested that chloride ion inhibition of GAD has evolved only in terrestial species which were not faced, like the lobster, with a high chloride ion concentration in their environment. Conversely insect GAD is similar to lobster and crayfish GAD in being stimulated by potassium ions (Baxter & Torralba, 1975; Chude et al., 1979; Breer & Heilgenberg, 1986; Stapleton, 1986; Molinoff & Kravitz, 1968; Wu et al., 1976). This effect has not been reported for vertebrate GAD.

GAD is thought to be involved in limiting the rate of GABA metabolism and therefore to be important in

the control of tissue concentrations of GABA. A refinement of this control is the possibility of feedback inhibition of GAD by GABA, since competitive inhibition of GAD by GABA has been reported in honeybee (Fox & Larsen, 1972), cockroach (Baxter & Torralba, 1975), locust (Breer & Heilgenberg, 1986), lobster (Molinoff & Kravitz, 1968) and crayfish (Wu et al., 1976). Baxter & Torralba (1975) have suggested that this feedback inhibition is a characteristic of the arthropod GAD which is not shared by the vertebrate enzyme. However human brain GAD is weakly inhibited by GABA (K_i= 10 mM, Blinderman et al., 1978). It is probable therefore that *in vivo*, insects are capable of GABA inhibition of GAD, but whether or not this effect is exclusive to the arthropod enzyme is unclear.

A further means of studying the similarities between enzymes from different species is by the use of antibodies. Su et al. (1983) have demonstrated that antibodies raised in rabbit against catfish GAD crossreact with goldfish, turtle, chick and frog GAD, but not with the mammalian enzyme. These antibodies also inhibit the GAD activity of *Drosophila* and crayfish (as well as frog, chick and goldfish) suggesting that the GAD enzymes of invertebrates and lower vertebrates are closely related to each other but not to the mammalian enzyme. In agreement with this Breer & Heilgenberg (1986) have observed low crossreactivity between antisera against rat GAD and the locust enzyme, although

they also observed strong crossreactivity of the antisera against rat GAD with trout GAD, which argues for a closer relationship between the GAD enzymes of higher and lower vertebrates than postulated by Su et al. (1983).

In summary there is ample evidence, in insects and crustaceans at least, that invertebrates possess GAD activity in their nervous tissues which has overall similarities with the well-characterized mammalian enzyme. However differences may exist between insect, crustacean and vertebrate GAD enzymes with respect to the inhibitory control of the enzyme and the affinity of the enzyme for its substrate. Though GAD is important in the control of GABA concentration in all these tissues, the fine tuning of the system probably differs, not only between vertebrates and invertebrates, but also between different invertebrate classes.For a more detailed review of this field, see Robinson & Olsen (1987).

1.5.2.2) THE METABOLISM OF GABA

In 1967 Hall & Kravitz (1967a) demonstrated GABA-T in extracts of lobster CNS. The enzyme is similar to other transaminases; it is stimulated by pyridoxal phosphate (PLP) and inhibited by carbonyl reagents such as hydroxylamine as well as high concentrations of its substrates, GABA and 2-oxoglutarate. Aminooxyacetic acid is the most potent inhibitor. The kinetic properties of

the enzyme are consistent with a substituted enzyme mechanism in which the product of the first substrate must leave the enzyme before the second substrate can bind. Other amino acids such as β-alanine and γ-amino valeric acid can act as substrates for the enzyme, but are not as good as GABA.

The same workers also characterized the lobster SSADH enzyme. It has an alkaline pH optimum of ~9, requires NAD^+ (though $NADP^+$ will also serve as cofactor), and also contains an active thiol group (2-mercaptoethanol and dithiothreitol activate the enzyme equally). In these respects the lobster SSADH is similar to the mammalian brain enzyme, but it differs in being inhibited by sodium chloride (Hall & Kravitz, 1967b). Both GABA-T and SSADH have been demonstrated in honeybee brain extracts (Fox & Larsen, 1972) and GABA-T has also been measured in locust and cockroach CNS (Sugden & Newsholme, 1977).

GABA-T purified from mouse brain is thought to be dimeric, having an M_r of 109k, and has a pH optimum of 8.05 (Schousboe et al., 1973). The Michaelis constants for GABA and 2-oxoglutarate are 1.1 mM and 0.25 mM respectively and the authors postulate therefore that the enzyme activity *in vivo* is probably not regulated by GABA concentration, but that 2-oxoglutarate may be important because its concentration is lower than its K_m. The mouse enzyme has a wide amino acid substrate specificity but narrow ketoacid substrate

specifity. Of a range of keto acids tested only 2-oxoglutarate served as an amino group acceptor, but several amino acids were capable of being donors. The donor needs to be a neutral ω-aminocarboxylic acid with the two functional groups separated by between two and four carbon atoms.

More recently GABA-T has been studied in the parasitic worm, Nippostrongylus brasiliensis (Watts & Atkins, 1984). The enzyme activity and K_m values (GABA, 0.33 mM; 2-oxoglutarate, 0.57 mM) are comparable with the mammalian enzyme. Glutamate inhibits the enzyme (K_i, 0.35 mM) and, as described above for purified mouse GABA-T, only 2-oxoglutarate acted as an amino group acceptor while a narrow range of amino acids were able to serve as donors. An unusual feature of the worm enzyme is that in a parallel isoelectric and chromatographic study with GABA-T from rat brain, while the rodent enzyme exibited a pI of 5.4, in agreement with other workers, the parasite enzyme has a pI of 10.5, implying that GABA-T from the nematode has a quite different amino acid composition from that of the enzyme from mammalian brain.

GABA-T has been detected in locust head and thoracic ganglia (Breer & Heilgenberg, 1985). More recently the enzyme has been purified in parallel from both sheep brain and whole locust heads (Jeffery, personal communication). Both the locust and sheep enzymes appeared as single proteins of identical size on

native polyacrylamide gel electrophoresis (PAGE); the M_rs of both native enzymes were 97k by gel permeation chromatography. In SDS-PAGE both enzymes migrated as a single species of M_r 50k, and are therefore thought to be dimeric, in agreement with other studies on mammalian GABA-T (Schousboe et al., 1973; John & Fowler, 1976). However, when a monoclonal antibody against rabbit brain GABA-T was used in immunoblotting studies, purified rabbit GABA-T showed a single band of M_r 52k while unfractionated rabbit brain supernatant showed a single band of 58k (Edwardson et al., 1985). These workers suggest therefore that the lengthy purification procedure may result in limited proteolysis of the enzyme and by analogy it is possible that the authentic insect GABA-T may have a marginally larger subunit size than reported by Jeffery (personal communication).

In the parallel study of Jeffery (personal communication), GABA-T from locust has a pH optimum of 8.3 and the K_m values for GABA and 2-oxoglutarate are comparable for the locust (0.8 mM and 0.27 mM respectively) and sheep (1.68 mM and 0.22 mM) enzymes. Also for both enzymes 2-oxoglutarate is the only effective amino group acceptor, as described above for GABA-T from other sources. Furthermore gabaculline is a potent suicide substrate (K_i~1-2 μM) for GABA-T from both sheep and locust. However the enzymes have different isoelectric points (locust, 6.65; sheep, 5.50) and antibodies raised in rabbit against sheep GABA-T show no

cross-reactivity with the locust enzyme in several different immunological studies. None of these antibodies interact with the active site of the enzyme though. Therefore, it seems possible that the GABA-T enzymes from insect and mammalian CNS have a very similar overall size and active sites, but that the amino acid composition of the enzymes differs, though this suggestion awaits the definitive evidence of amino acid analyses of the enzymes.

Within the invertebrates there is now very good evidence that insects, crustaceans and nematodes possess the enzymic machinery for the metabolism of GABA. Furthermore, the major metabolic enzyme, GABA-T, appears very similar in size, subunit composition, and catalytic characteristics in both invertebrate and vertebrate CNS, though major differences may exist in the amino acid composition of the enzyme from different species.

1.5.3/ Studies on GABA uptake

Once GABA has been released from the nerve terminal and interacted with its post-synaptic receptor it is necessary that the resulting signal is then terminated. Though nervous tissues are known to contain the enzymes necessary for the metabolism of GABA, it is thought that GABA-T is not directly responsible for the removal of GABA from the synaptic cleft. Since it requires 2-oxoglutarate as a substrate, GABA-T is

unlikely to be located outside the nerve endings, and furthermore GABA-T inhibitors do not have any prolonging effect on neuromuscular transmission in the lobster (Hall & Kravitz, 1967a). Instead GABA is thought to be removed from the synapse by neuronal and glial uptake.

One of the first demonstrations of such uptake was in an invertebrate nerve-muscle preparation. Iversen & Kravitz (1966, 1968) found that on incubating the ventral superficial abdominal muscles of the lobster in 20 nM [^{3}H]GABA, the preparation accumulated four to five times the concentration of radioactivity in the medium, and that 90% of this was still in the form of GABA. This uptake was sodium-dependent, exhibited a K_m of 6×10^{-5} M, and was inhibited by β-guanidinopropionic acid, γ-hydroxy GABA, desipramine and chlorpromazine (all at 10^{-3} M). Furthermore these workers demonstrated that GABA could be released by stimulation of a single inhibitory axon innervating lobster muscle (Kravitz et al., 1968). More recently Craelius & Fricke (1981) have shown that the inhibitory neurones innervating the muscle receptor organ of crayfish, following exposure to [^{3}H]GABA, will release the radioactivity in response to low frequency electrical stimulation, in a Ca^{2+}-dependent manner. Subsequent to the early work of Iversen & Kraviz (1966), Iversen & Neal (1968) demonstrated the active accumulation of GABA by slices of rat cerebral cortex, and 70% of [^{3}H]GABA uptake by slices and homogenates of various brain regions was

shown by autoradiography to be localized in the nerve terminals (Iversen & Bloom, 1972). Sodium-dependent GABA uptake was also demonstrated in synaptosomes from mouse brain (Kuriyama et al., 1969) and rat brain (Martin & Smith, 1972). Indeed the availability of mammalian synaptosomes opened the doors to several extensive studies of GABA uptake such that it became widely accepted that neurones (and glial cells, see Enna & Snyder, 1975; De Feudis, 1979) of mammalian CNS are capable of GABA uptake. Subsequently it was possible to obtain both Ca^{2+}-dependent and Ca^{2+}-independent, K^{+}-evoked GABA release from synaptosomes. However there is still controversy as to whether or not Ca^{2+}-dependent release is from cytosolic (eg. De Belleroche & Bradford, 1977) or non-cytosolic compartments (Haycock et al., 1978).

It should be noted that in the early studies it was thought that GABA uptake was directly responsible for the termination of the response. However Eccles & Jaeger (1958) had demonstrated mathematically that the time course of diffusion of transmitter from the synaptic cleft was sufficiently rapid to account for transmitter inactivation. Evidence has recently been provided in support of this for GABA inactivation in crayfish stretch receptor neurones (Deisz et al., 1984). Bath applied GABA causes a conductance increase in voltage-clamped neurones which is enhanced in magnitude, but not in its time course, by the uptake inhibitor,

nipecotic acid. Furthermore rapid conductance increases were only observed at high GABA concentrations, leading the authors to postulate that the prominent action of GABA uptake is to maintain a low cleft concentration of GABA rather than to terminate the response.

Following the demonstration of GABA uptake in lobster muscle (Iversen & Kravitz, 1966, 1968), while work on mammalian synaptosomal GABA uptake and release was making rapid progress, invertebrate investigators had to wait fourteen years before they had synaptosomes available to them. However, it was possible to make autoradiographic and electrophysiological studies of invertebrate [^{3}H]GABA uptake.

Frontali & Pierantoni (1973) incubated brain slices from the cockroach, Periplaneta americana, with [^{3}H]GABA and demonstrated that GABA accumulated over certain neurones and/or their processes or endings, while much longer times of autoradiograph exposure were required to detect low levels of glial uptake. In an electrophysiological study on the abductor muscle of crayfish, Horvitz and Orkand (1978) reported a GABA uptake mechanism which was blocked by nipecotic acid and L-2,4-diaminobutyric acid. However this mechanism was not Na^+-dependent, which the authors attribute to the relatively high concentrations (10^{-5} M) of GABA employed in the study. In a more recent autoradiographic study (Hue et al., 1982), the two last abdominal ganglia from the cockroach were incubated in a saline containing

[^{3}H]GABA. The transmitter accumulated at the periphery of the ganglia, and uptake of GABA into the terminal ganglion was partially inhibited by sodium-free medium. Because the uptake was also inhibited by 10 mM β-alanine, this observed GABA uptake mechanism was thought to be at least partially glial, but more recently Griffiths et al. (1986) have shown that β-alanine is not specific for glial GABA uptake systems. Inhibitory motor neurones of the leech CNS have been shown to take up [^{3}H]GABA and this uptake is stimulated by electrical activity of the neurones (Cline et al., 1985). Van Marle et al. (1985) have made an autoradiographic investigation of GABA uptake into two morphologically distinct insect neuromuscular synapses; the common inhibitor innervated slow muscle exhibits [^{3}H]GABA uptake by both glia and terminal axons, while the slow extensor tibia innervated only exhibits glial uptake. Shepherd & Tyrer (1985) have also demonstrated a GABA uptake mechanism electrophysiologically in insect skeletal muscle, and they conclude that this may be glial, though like many invertebrate studies, this conclusion is based largely on mammalian pharmacology.

Crayfish muscle plasma membrane vesicles have been shown to accumulate GABA at 0^{o}C in a process dependent on sodium ions and membrane integrity (Meiners et al., 1979). The process was saturable with a K_m of 0.55 μM which is much lower than that reported for intact lobster muscle at 20^{o}C (K_m=60 μM, Iversen & Kravitz,

1966) or crayfish muscle slices at 22°C (22 μM, Olsen et al., 1975). These differences may be due to the use of isolated vesicles as against slices or intact neuromuscular tissue. Alternatively an additional low affinity uptake system may exist in the vesicular preparation. The vesicular uptake of [^{3}H]GABA (Meiners et al., 1979) is inhibited by both nipecotic acid (IC_{50}=0.45 μM) and slightly more potently by the mammalian neuronal inhibitor 2,4-diaminobutyric acid (DABA) (IC_{50}=0.3 μM). However, even at 1 mM neither of these ligands inhibited the uptake by more than 80%, supporting the possibility of a secondary uptake system. It therefore seemed likely that uptake was involved in the removal of GABA from the crustacean neuromuscular junction, but claims for the existence of any similar uptake system in the invertebrate CNS relied solely on the autoradiographic evidence described above.

In 1980 Breer & Jeserich published a method for the production of synaptosomes from insect CNS. However invertebrate synaptosomes have only seen very limited use in the study of GABA uptake and the little work that has been done (Gordon et al., 1982; Breer & Heilgenberg, 1985; Whitton et al., 1986) will be discussed in Chapter 3 in comparison with the data presented in this thesis. However the intimation from the various studies described above is that in addition to invertebrate neuromuscular junctions, insect (and annelid) CNS possesses GABA uptake systems which may

well play a role in the removal of GABA from the synapse.

1.5.4/ GABA receptors

Much of the early evidence for GABA receptors in nervous tissues came from electrophysiological studies in invertebrate systems (see Gerschenfeld, 1973). Owing to such factors as the ease of obtaining large quantities of tissue though, the subsequent biochemical characterization of the GABA receptors involved in neurotransmission was carried out with mammalian brain. However, as we will see later, in the last five years there has been an upsurge of interest in invertebrate GABA receptors, and the evidence for GABA receptors in invertebrates will be compared in Chapter 2 with our knowledge of vertebrate GABA receptors and the data presented in this thesis.

CHAPTER 2

INSECT CENTRAL GABA RECEPTORS

2.1/ INTRODUCTION

It was shown in Chapter 1 that much of the early evidence for the presence of GABA receptors in nervous tissues came from electrophysiological studies in invertebrate systems (see Gerschenfeld, 1973). However the biochemical characterization of receptors involved in GABAergic neurotransmission has been carried out in mammalian brain and therefore any subsequent study of invertebrate GABA receptors must be conducted in comparison with what is already known of the mammalian GABA receptors.

2.1.1/ Mammalian GABA receptors

The Na^+-independent, bicuculline-sensitive binding of [^{3}H]GABA to mammalian brain fractions was first demonstrated by Zukin et al. (1974), and was subsequently characterized by several groups (Enna & Snyder, 1975; Greenlee et al, 1978b; Krogsgaard-Larsen et al., 1979). It is now known that there are at least two classes of GABA receptors, $GABA_A$ and $GABA_B$, in mammalian brain. $GABA_A$ receptors activate Cl^- channels and are sensitive to bicuculline, while $GABA_B$ receptors modulate Ca^{2+} channels and are sensitive to baclofen. $GABA_B$ receptors may have a presynaptic location and they are linked to adenylate cyclase via guanine nucleotide binding proteins. Hence the binding of GABA to $GABA_B$

receptors inhibits adenylate cyclase activity and thereby depresses the intracellular concentration of cAMP (Bowery et al., 1984; Hill et al., 1984).

2.1.1.1/ THE MAMMALIAN $GABA_A$ RECEPTOR COMPLEX

Based on radioligand binding studies, the $GABA_A$ receptor has been shown to be a complex of a chloride channel associated with interacting binding sites for 1/ GABA, 2/ the benzodiazepines and 3/ picrotoxin, cage-convulsants, such as t-butyrylbicyclophosphorothionate (TBPS), and the barbiturates. This latter group of compounds appear to interact more closely with the chloride channel of the receptor than the former two. The results of the numerous binding studies carried out on the complex have been extensively reviewed (Olsen, 1981; Olsen, 1982; Braestrup & Nielsen, 1983; Haefely, 1984; Tallman & Gallager, 1985; Karobath & Supavilai, 1985; Fischer & Olsen, 1986) and the complex is depicted in a highly simplified model in Fig.3a (after Olsen, 1981), though it should be noted that more complex models have since been postulated (eg. Haefely, 1984).

In addition to radioligand binding studies, the benzodiazepine receptor on the $GABA_A$ receptor complex has also been studied by photoaffinity labelling whereby [^{3}H]flunitrazepam is covalently attached to the receptor by irradiation with ultra-violet light (Mohler

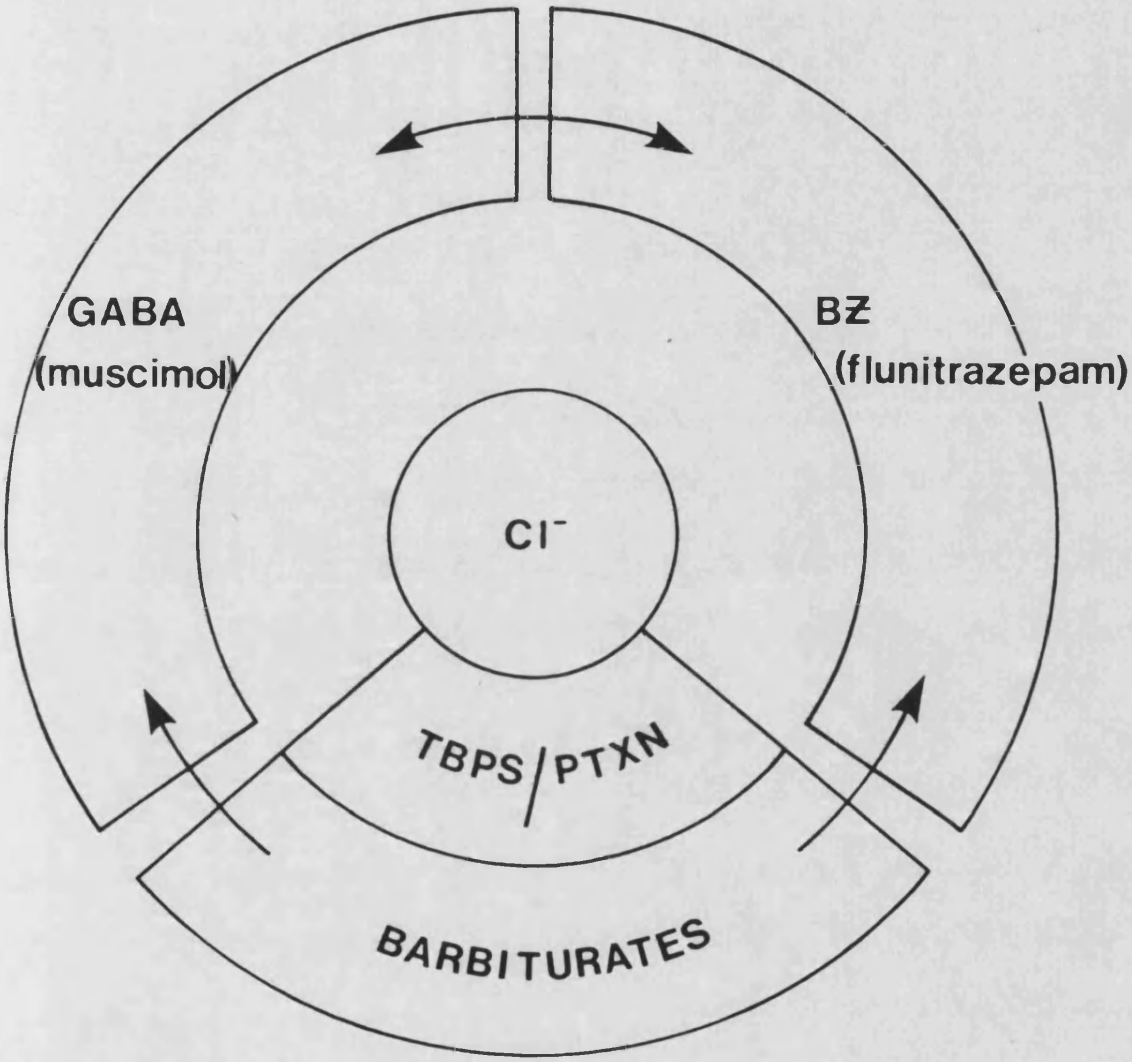


Fig.3a Simplified model of the mammalian $GABA_A$ receptor complex, after Olsen (1981), showing interacting binding sites for GABA, the benzodiazepines (BZ) and picrotoxin (PTXN) and cage convulsants (TBPS) asociated with a Cl^- ion channel.

et al., 1980). This technique has demonstrated that the peptide carrying the benzodiazepine receptor has an M_r of 50,000. However, there is evidence that in addition to the mammalian benzodiazepine receptors that are associated with a $GABA_A$ receptor, other, less well-characterized, benzodiazepine receptors are present in mammalian CNS and this topic will be discussed in more detail later in this chapter.

All the binding activities of the mammalian $GABA_A$ receptor have been co-purified as one complex from both bovine and porcine brain by extraction from the membrane with detergents and subsequent affinity chromatography using benzodiazepine affinity resins (Sigel et al., 1983; Olsen et al., 1984; Sigel & Barnard, 1984; Kirkness & Turner, 1986a). Furthermore, the purified complex has been reconstituted into phospholipid vesicles with retention of its binding activities (Sigel et al., 1985). The purified complex possesses two subunits with M_r values of 50k and 55k, and the target size for benzodiazepine and GABA binding in membranes when determined by radiation inactivation was 220k (Chang & Barnard, 1982). These data imply a tetrameric model for the receptor. This was also indicated by photoaffinity labelling experiments in which photoreaction of one binding site in the membrane caused a conformational change in three additional sites such that only 25% of the benzodiazepine receptors could be photoaffinity labelled (Mohler et al., 1980; Thomas &

Tallman, 1981; Karobath & Supavilai, 1982). More recently, polyclonal (Stephenson et al., 1986) and sixteen monoclonal antibodies (mAbs) (Schoch et al., 1985) have been raised to the purified complex. Two of the mAbs (bd-17 and bd-24) gave the same results in immunoprecipitation studies with purified bovine receptor in that they both co-precipitated all the high and low affinity GABA binding sites as well as the binding sites for the benzodiazepines and TBPS. However in immunoblotting experiments bd-17 and bd-24 behaved differently. On denaturing electrophoretic gels of purified bovine receptor, bd-17 only recognized the β-subunit (M_r 55k) while bd-24 only recognized the α-subunit (M_r 50k). Therefore it was concluded that these antibodies recognize epitopes on different subunits but immunoprecipitate the entire GABA receptor complex and therefore each receptor is heterogeneous with respect to its subunit composition (Haring et al., 1985; Mohler et al., 1986). The purification, labelling and immunological studies on the mammalian $GABA_A$ complex have led Mohler et al. (1986) to postulate the $\alpha_2\beta_2$ tetrameric model shown in Fig.3b as a preliminary working model of the complex. The benzodiazepine binding sites definitely reside on the α-subunit which is recognized by MABII (bd-24), but may also occur on the β-subunit in a state which is not generally photoaffinity labelled. The location of the GABA sites is less clear. By [^{3}H]muscimol photoaffinity labelling,

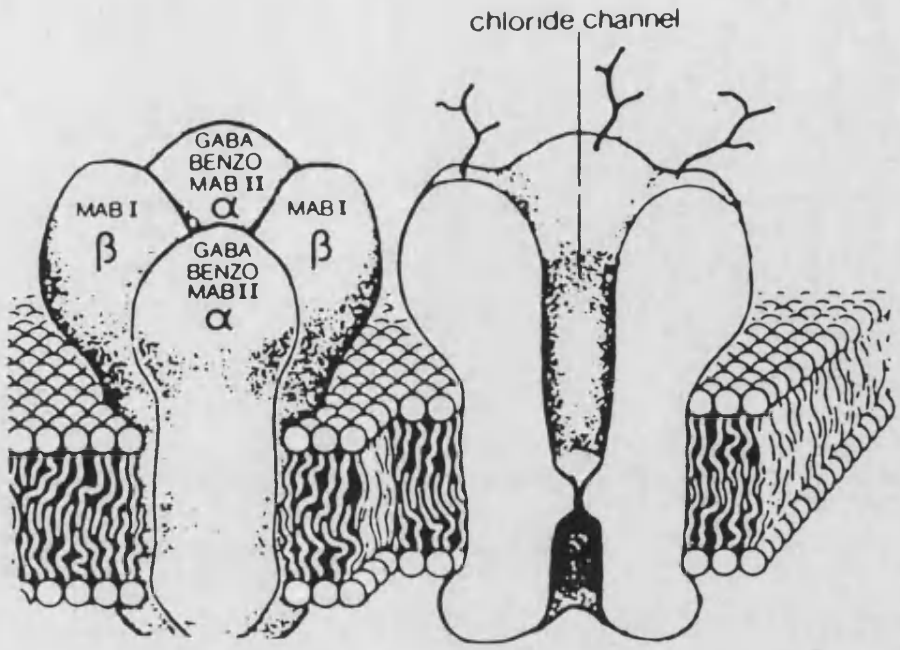


Fig.3b Preliminary working model of the mammalian $GABA_A$ receptor complex postulated by Mohler et al. (1986). The complex is composed of four subunits ($\alpha_2\beta_2$) with carbohydrate moieties as indicated by the branched lines. The α-subunit possesses binding sites for the benzodiazepines (BENZO) and possibly for GABA. The α- and β-subunits can be distinguished between by monoclonal antibodies (MAB I & II) as described in the text. The subunits are associated together such that they form a Cl^- channel through the membrane.

GABA sites were assigned to the 50k protein and hence to the α-subunit (Asano et al., 1983; Cavalla & Neff, 1985) and more recently this has been confirmed using purified receptor complex from porcine brain (Kirkness & Turner, 1986b). However Deng et al. (1986) have performed a photoaffinity labelling study on purified bovine brain receptor complex, using both [^{3}H]FNZP and [^{3}H]muscimol and while a 52k (α) subunit was labelled by the benzodiazepine, [^{3}H]muscimol labelled the 57k (β) subunit. [^{3}H]Muscimol has also been reported to photoaffinity label the β-rather than the α-subunit of the purified receptor complex of bovine brain by Casalotti et al. (1986). The reasons for these discrepancies in the subunit allocation of the GABA site are unclear.

A further consideration which has not been clarified is the location of the TBPS binding site. An $\alpha_2\beta_2$ tetramer may form a Cl^- channel which binds TBPS. However in purified preparations of $GABA_A$ receptor complex an additional peptide of M_r ~60k has been reported (Schoch et al., 1984; Sigel & Barnard, 1984; Olsen et al., 1984) and the target size of the radiation inactivated TBPS binding site was 134k (Nielsen & Braestrup, 1983). Furthermore receptor aggregates possessing TBPS allosteric interactions have been reported to have a radiation inactivation size of 350k (Nielsen & Breastrup, 1984), leading Mohler et al. (1986) to make the reservation that their model shown in

Fig.3b may need modifying to include an additional subunit (γ) of M_r 62-80k which binds TBPS. Though the precise subunit composition of the mammalian $GABA_A$ receptor complex, and the assignment of binding sites to these subunits, has not been established yet, this goal should be achieved in the near future. Indeed, information is already being obtained on the amino acid sequence of the receptor by the application of cloning techniques (Mohler, 1986), and functional GABA receptor complexes have already been expressed in Xenopus oocytes by injection of chick brain mRNA (Smart et al., 1983).

2.1.2/ The heterogeneity of mammalian benzodiazepine receptors

The benzodiazepine receptors described so far in this chapter have been central neuronal benzodiazepine receptors (CBRs) which are largely associated with $GABA_A$ receptors as described and which are sensitive to the benzodiazepine clonazepam. However a second type of binding site exists on non-neuronal cells which is sensitive to the benzodiazepine Ro5-4864 rather than clonazepam. These benzodiazepine binding sites were first detected on kidney and are hence referred to as peripheral benzodiazepine receptors (PBRs) (Regan et al., 1981) and will be described in more detail later. A third benzodiazepine site of much lower affinity has been described in mammalian brain (Bowling & De Lorenzo, 1982) which appears to be associated with

the calcium-calmodulin protein kinase system in the brain (De Lorenzo, 1981). The localization and function of these sites is obscure.

2.1.2.1/ CENTRAL BENZODIAZEPINE RECEPTORS

1/ Agonists, antagonists and inverse agonists

The clonazepam-sensitive CBRs are sensitive to several different classes of ligand which not only affect the CBRs differently, but also elicit different actions to the benzodiazepines when administered to animals (see Tallman & Gallager, 1985). Essentially the agonistic benzodiazepines such as diazepam enhance GABA binding by decreasing the K_D of the GABA receptor for its ligand. When administered to animals these compounds therefore have anxiolytic, anti-convulsant, sedative and muscle relaxant effects. However a second class of ligands, the β-carbolines, inhibit benzodiazepine binding but have an action opposite to that of the agonistic benzodiazepines in that they increase anxiety and convulsions when administered to animals. Such ligands are referred to as inverse agonists. Another group of compounds, the imidazodiazepines, of which the most potent is Ro15-1788, block both benzodiazepine and β-carboline binding to brain membranes. However these antagonists have no pharmacological activity when administered to animals alone. This complex pharmacology

has been rationalized by Ehlert (1986), based on a model in which the benzodiazepine receptor exists in two states, dependent on whether the associated Cl^- channel is open or closed, and different ligands have different selectivities for the two conformational states. Hence benzodiazepines such as diazepam are selective for the open state of the channel and therefore enhance GABA-induced Cl^- influx, whereas the β-carbolines bind to the closed state and therefore have opposite pharmacological effects to the tranquilizer benzodiazepines. Other compounds, such as Ro15-1788, though potently binding to the benzodiazepine receptor, have no selectivity for the open or closed states, and therefore, though inhibiting the binding of other ligands, they have no pharmacological activity of their own in whole animal studies.

2/ Type I and Type II central benzodiazepine receptors

An additional class of ligands acting at the CBR are the triazolpyridines, such as CL 218,872, which are also anxiolytic without being sedative. However, CL 218,872 is a partial agonist in that it does not inhibit the binding at all benzodiazepine receptors, and indeed it is only active on CBRs in discrete brain regions (Klepner et al., 1979). These workers claimed that this reflected a true heterogeneity of CBRs and designated the CL

218,872-sensitive receptors Type I and the triazolopyridine-insensitive CBRs as Type II. Type I receptors were postulated to be responsible for the anxiolytic activity of the benzodiazepines while the Type II receptors mediate their sedative actions.

However, Gee & Yamamura (1982) and Johnson et al. (1983) pointed out that the brain regions where the Type I (eg: cerebellum) and Type II (eg: hippocampus) receptors were located did not coincide with the regions thought to be involved in anxiety. Such considerations led these workers to suggest that benzodiazepine receptor Types I and II correspond to different conformational states of a structurally homogeneous protein. However Lippa et al. (1985) and Hirsch et al. (1985) maintain that there is a true heterogeneity of benzodiazepine receptors at the level of the constituent subunits, based on considerations of regional B_{max} values for the binding of [^{3}H]β-carboline (which also acts at the Type I receptor) and from autoradiographic studies. Heterogeneity is also apparent in the regional pattern of membrane photolabelling with [^{3}H]FNZP. With this technique a protein of M_r 50k was found in all brain regions (Mohler et al., 1980; Sieghart & Karobath, 1980; Thomas & Tallman, 1981; Sieghart & Drexler, 1983; Mohler et al., 1984). However, principally in hippocampus, an additional protein of M_r 55k was also labelled (Sieghart & Karobath, 1980; Sieghart & Drexler, 1983; Mohler et al., 1984; Eichinger & Sieghart, 1984),

and triazolopyridines and β-carbolines are 10-fold more potent in inhibiting the labelling of the 51k protein than the 55k protein in hippocampus (Sieghart et al., 1983). These observations led Hirsch et al. (1985) to postulate that the peptide of M_r 51k possesses the Type I receptor while that of M_r 55k possesses the Type II receptor.

As described above, mAbs which differentiate between the α-subunit (M_r 50k) and the β-subunit (M_r 55k) are now available (Schoch et al., 1985). The use of these antibodies has led Mohler et al. (1986) to refute such a clear cut heterogeneity as postulated by Hirsch et al. (1985). All areas, including cerebellum, exhibit the β-subunit ("Type II" CBR) by immunoblotting, and immunostaining of brain sections with mAbs to either subunit gave the same picture. Therefore differences in the profiles of benzodiazepine receptor ligands cannot be attributed to a major structural heterogeneity of the receptor complex, though the antibody studies do not rule out the possibility of conformational heterogeneity. A further possibility which might explain the regional differences in photoaffinity labelling is that both the α- and β-subunits bind benzodiazepines, but whereas normally the β-subunit cannot be photolabelled, the hippocampal β-subunit is sufficiently different to permit such labelling to occur.

What is clear is that mammalian central benzodiazepine receptors, on extensive analysis, have

revealed a highly complex picture that has still to be fully elucidated.

2.1.2.2/ PERIPHERAL BENZODIAZEPINE RECEPTORS

In addition to the binding of benzodiazepines to mammalian central benzodiazepine receptors (CBRs), [^{3}H]FNZP has also been observed to bind to peripheral benzodiazepine receptors (PBRs) on rat kidney membranes (Regan et al., 1981). The CBRs are sensitive to clonazepam whereas the PBRs are sensitive to Ro5-4864. In addition to the localization of PBRs on peripheral tissues, the availability of [^{3}H]Ro5-4864 has enabled the demonstration of PBRs in rat brain (Schoemaker et al., 1981), spinal cord (Villiger, 1985) and rat striatal muscle (Muller et al., 1985). However the binding of Ro5-4864 to brain PBRs is not affected by GABA, and in further contrast with CBRs, the brain PBRs cannot be photoaffinity labelled (Marangos et al., 1982). The PBRs have also been claimed to differ from the CBRs of mammalian brain in their subcellular localization, being located primarily in the nuclear rather than synaptosomal fraction on subcellular fractionation of brain tissue (Marangos et al., 1982). Weissman et al. (1984a) have reported four-fold higher concentrations of PBRs in guinea pig brain compared with rat brain, and they support the nuclear localization of PBRs reported by Marangos et al. (1982). However it

should be noted that Basile & Skolnick (1986) have disputed this and claim that PBRs are enriched in mitochondrial and synaptosomal rather than nuclear membranes. The density of PBRs is greater on mitochondrial than on synaptosomal membranes while this distribution is reversed for CBRs. A final difference between brain PBRs and CBRs may lie in their regional localization. In an autoradiographic study of [^{3}H]Ro5-4864 binding sites, Gehlert et al. (1985) reported PBRs to exhibit their highest density in the choroid plexus and eppendymal cells (which are associated with the blood brain barrier) which is in contrast with the localization of CBRs. The major differences between CBRs and PBRs are summarized in Table 3.

In their autoradiographic study of PBRs, Gehlert et al. (1985) also reported that the PBRs of kidney were associated with the ascending Loop of Henle and therefore postulated a physiological role for these receptors in ion transport. However the peripherally-active benzodiazepine, Ro5-4864, has been shown to be anxiogenic and produce convulsions in various mammals and to have central pharmacological effects which are the opposite of those of the tranquilizer benzodiazepines active at the central $GABA_A$ receptor complex (Pierri et al., 1983; Polc et al., 1983; Weissman et al., 1984b; File & Pellow, 1985). Furthermore, the Ro5-4864-elicited convulsions in mice

CHARACTERISTIC	PERIPHERAL-TYPE	CENTRAL-TYPE
TISSUE SOURCE	NEURONAL & OTHER TISSUES EG: KIDNEY	NEURONAL
LIGAND SENSITIVITY	Ro5-4864 - SENSITIVE	CLONAZEPAM - SENSITIVE
EFFECT OF GABA	UNAFFECTED BY GABA	ENHANCED BY GABA
PHOTOAFFINITY LABELLING	CAN NOT BE PHOTOLABELLED WITH FLUNITRAZEPAM	CAN BE PHOTO-LABELLED WITH FLUNITRAZEPAM

Table 3 Summary of the major differences between mammalian peripheral- and central-type benzodiazepine receptors.

are inhibited by the central GABAergic ligands diazepam, clonazepam, muscimol and pentobarbital, but the CBR antagonist, Ro15-1788, was inactive against the convulsions, implying some perturbation of the central $GABA_A$ receptor complex (Weissman et al., 1984b). Ro5-4864-induced convulsions in rats have also been reported to be blocked by diazepam, clonazepam and pentobarbital, while the GABA antagonist, bicuculline, enhances the convulsions (Rastogi & Ticku, 1985).

In addition to behavioural studies, both electrophysiological and biochemical studies have been made on the central actions of Ro5-4864. This peripherally-active benzodiazepine has been shown to inhibit the GABA-mediated excitatory effects of flurazepam on the firing rates of dopaminergic neurones in rat substantia nigra (MacNeil et al., 1983). Furthermore Ro5-4864 blocks the potentiation of muscimol-evoked depolarization in slices of rat cuneate nucleus by both flurazepam and pentobarbital. Ro5-4864 also potentiated the antagonism of muscimol responses by picrotoxin (Simmonds, 1985).

MacNeil et al. (1983) also reported that Ro5-4864 inhibits the GABA enhancement of [^{3}H]diazepam binding to rat cortex. In addition, Ro5-4864 has been reported to competitively inhibit the binding of the cage convulsant [^{35}S]TBPS to membranes from rat cortex and cerebellum, though the inhibition is unaffected by central GABAergic ligands (Ticku & Ramanjaneyulu, 1984).

From these various behavioural, electrophysiological and biochemical studies, several workers have claimed that Ro5-4864 elicits its pharmacological effects in the mammalian brain by impairing central GABAergic neurotransmission. Rastogi & Ticku (1985) go as far as to suggest that these effects may be via a direct interaction of Ro5-4864 with the picrotoxin site on the $GABA_A$ receptor complex, but others suggest that the action of Ro5-4864 is either via some other site on the complex (File & Pellow, 1985) or an indirect effect on the complex (Weissman et al., 1984b). Whether or not the convulsant effects of Ro5-4864 are via the high affinity [^{3}H]Ro5-4864 binding sites in mammalian brain is not clear, though the autoradiographic localization of these sites reported by Gehlert et al. (1985) make this unlikely. What this brief review of the literature on peripheral-type benzodiazepine activity does illustrate is that from an initial detection of Ro5-4864-sensitive benzodiazepine binding sites which were assumed to be merely acceptors, of no known function, our knowledge of peripheral-type benzodiazepine activity has expanded such that we now have a very complex pharmacological picture which has still to be clarified.

2.1.3/ Insect central GABA receptors

Despite our extensive knowledge of mammalian central GABA and benzodiazepine receptors, until recently there was virtually no information available

regarding any corresponding receptors in insect CNS. In a phylogenetic study of [^{3}H]diazepam binding activity, Nielsen et al. (1978) reported that the head ganglia of five invertebrate species (including the locust) lacked benzodiazepine receptors. These authors concluded that brain-specific benzodiazepine receptors had a late evolutionary appearance which coincided with the development of the higher boney fishes. In a second phylogenetic study, Mann & Enna (1980) reported that cockroach ganglia exhibited less than 10% displacement of [^{3}H]GABA binding by 0.1 mM bicuculline, and they postulated that the evolution of bicuculline-sensitive GABA receptors occurred with the appearance of the vertebrates. Thus it appeared that any GABA receptors in insect CNS were bicuculline-insensitive and did not have associated benzodiazepine receptors. Based on limited studies (largely in crustacean muscle and molluscan neurons), Simmonds (1983) postulated that invertebrate GABA receptors in general, though sensitive to muscimol and picrotoxin, are insensitive to bicuculline, 3-APS, benzodiazepines and pentobarbital, and were therefore very different from the complex $GABA_A$ receptors of mammalian brain.

The first positive insect GABAergic binding study was reported by Abalis et al. (1983) who measured the binding of [^{3}H]FNZP to housefly thoracic muscle membranes. Though the authors could not detect [^{3}H]muscimol or [^{3}H]GABA binding to the same membranes,

the benzodiazepine binding was enhanced by GABA, implying a GABA/benzodiazepine linkage in these tissues. However there were still no reports on the GABA receptors of insect CNS, and therefore a study was made of [^{3}H]muscimol and [^{3}H]FNZP binding sites in locust CNS. The results of these studies are presented in this chapter and are discussed in parallel with the more recent results of other studies of GABAergic binding activities in insect central tissues.

2.2) MATERIALS & METHODS

MATERIALS

All reagents were obtained from Sigma Chemical Corporation or BDH Chemicals Ltd unless otherwise stated. Two-week old adult locusts (Schistocerca gregaria), in the 5th instar, were used in all experiments. Locusts were supplied by the Welsh Mountain Zoo, Colwyn Bay, Wales. [^{3}H]Muscimol (29.4 Ci/mmol) was obtained from New England Nuclear Corporation (Boston, USA). [^{3}H]Flunitrazepam (84 Ci/mmol or 74 Ci/mmol) was obtained from Amersham International plc (England). Cold benzodiazepines were a gift from Hoffmann-La Roche Ltd. The GABA agonists muscimol and isoguvacine HCl were obtained from Cambridge Research Biochemicals Ltd., England. Soluene tissue solubilizer was obtained from the Packard Instrument Co.

All measurements of radioactivity were made in tolune scintillant containing 30% (v/v) Triton X-100 and 0.5% (w/v) PPO, in either an LKB 1217 Rackbeta liquid scintillation counter or a Packard Minaxi Tri-Carb 4000 Series liquid scintillation counter, using a 2 minute counting period unless stated otherwise. The counting efficiency was 35% and quenching was corrected for.

METHODS

2.2.1/ Preparation of the synaptosomal membrane fraction, P_2G, from locust supraoesophageal ganglion

The insects were decapitated and the heads stored on ice. The cuticle of the head was shaved off, from the mouthparts to the top of the head, to expose the yellow supraoesophageal ganglion (Fig.4). The ganglia were removed into 10 mM TrisHCl, pH 7.4, containing 0.25 M sucrose and 1 mM EGTA, at 4°C (tissue from 50 heads/5 ml). The suspension was homogenized (7 x 10 strokes) in a Potter-Elvehjem homogenizer with a motor driven pestle (speed, 500 rpm; radial clearance, 0.15 mm). The resulting homogenate was fractionated according to the scheme shown in Fig.5.

2.2.2/ Preparation of the synaptosomal membrane fraction P_2R from rat brain

The membrane fraction P_2R from rat brain was prepared as described by Braestrup & Squires (1977). Fresh whole rat brains were homogenized (7 x 10 strokes) in 0.32 M sucrose, containing 1 mM EDTA (10 ml/brain), in a Potter-Elvehjem homogenizer, 0.15 mm radial clearance. The homogenate was centrifuged at 2000 x g for 5 min at 5°C, and the resulting supernatant was removed and centrifuged at 30,000 x g for 10 min at 5°C. The resulting pellet, P_2R, was resuspended in assay buffer (10 mg original tissue/400 µl).

Fig.4 View of the top of the head of the locust with the cuticle shaved away to expose the supraoesophageal ganglion, which is shown dissected out below. Scale is in mm.

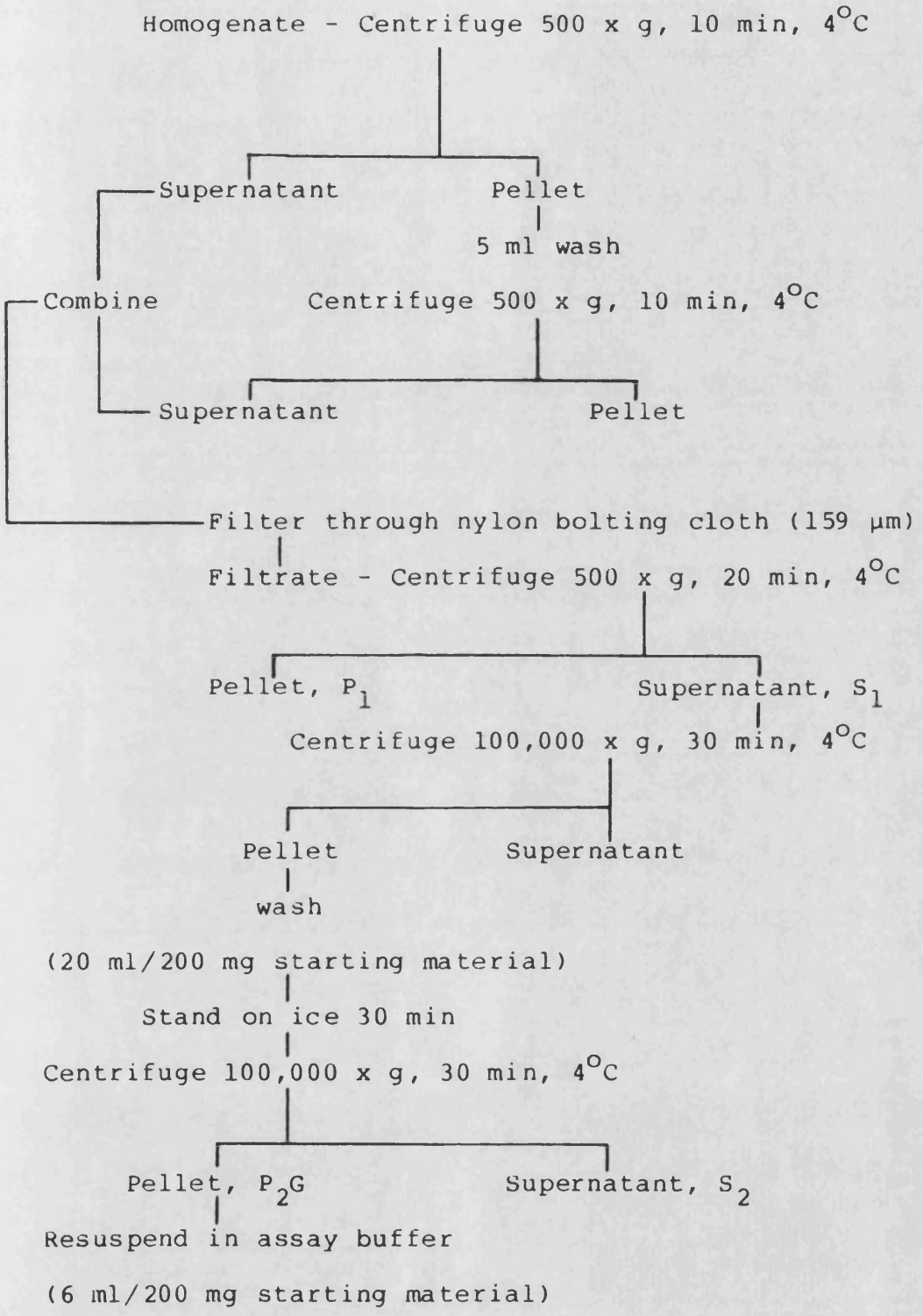


Fig.5 Fractionation scheme for locust supraoesophageal ganglia

2.2.3/ Assay of [^{3}H]muscimol binding in P_2G

All assays of [^{3}H]muscimol binding in P_2G were made in triplicate by the centrifugation method of Meiners et al. (1979). Resuspended membranes (400 µl, Method 2.2.1) were added to 50 µl assay buffer $\pm 10^{-3}$ M GABA plus 50 µl [^{3}H]muscimol in assay buffer (10 mM TrisHCl, pH 7.4, containing 0.15 M choline chloride and 1 mM EGTA) (10 x required final concentration) in an Eppendorf microcentrifuge tube. The mixture was incubated for 30 min at 4^{o}C, and then centrifuged at 11,500 x g for 5 min in an MSE Micro Centaur microcentrifuge. The resulting pellet was washed twice with 1.5 ml ice-cold assay buffer. Following overnight solubilization of the pellet in Soluene tissue solubilizer (75 µl) the mixture was neutralized with an equal volume of glacial acetic acid and the microcentrifuge tube bottom and contents were removed into a counting vial with a hot scalpel blade. Scintillant (5 ml) was added and the radioactivity was counted. Non-specific binding (binding in the presence of 10^{-4} M GABA) was subtracted from the total binding (binding in the absence of 10^{-4} M GABA) to give a measure of specific binding.

The effects of various ligands on [^{3}H]muscimol binding were investigated in P_2G in the same manner except that 10^{-3} M GABA was replaced by test ligand at the required concentration. When the effect of Na^+ ions on [^{3}H]muscimol binding was investigated in P_2G, the

assay buffer contained 0.15 M NaCl and 1 mM EDTA rather than 0.15 M choline chloride and 1 mM EGTA.

2.2.4/ Assay of [^{3}H]flunitrazepam binding in P_2G and P_2R

All assays of [^{3}H]flunitrazepam binding in P_2G and P_2R were made by the filtration method of Braestrup & Squires (1977). The assay buffer used initially was the same as that described for the [^{3}H]muscimol binding assays (Method 2.2.3). However, this was subsequently replaced by 10 mM TrisHCl, pH 7.4, containing 0.15 M choline chloride, 4 mM $CaCl_2$, and 4 mM $MgCl_2$ (see Results 2.3.1-5). Resuspended membranes (400 µl, Methods 2.2.1 & 2) were added to 50 µl assay buffer $\pm 10^{-3}$ M diazepam plus 50 µl [^{3}H]flunitrazepam in assay buffer (10 x required final concentration of [^{3}H]FNZP) to give a total assay volume of 500 µl. The mixture was incubated for 30 min at 4°C and then applied to a Whatman GF/C filter (2.5 cm) under vacuum. The filter was washed with 10 ml ice-cold assay buffer, dried in an oven for 15 min, and then placed in a scintillation vial with 5 ml scintillant and the radioactivity counted. Specific binding was taken as the difference in the binding in the absence and the presence of 10^{-4} M diazepam. To investigate the effects of various ligands on [^{3}H]FNZP binding, 10^{-3} M diazepam was replaced by test ligand.

2.2.5/ Measurement of calcium and magnesium by atomic absorption spectroscopy

The concentrations of calcium and magnesium in rat brain, locust ganglion, and the membrane fractions P_2R and P_2G prepared from these tissues, were measured by atomic absorption spectroscopy.

Portions of tissue (40-500 mg wet weight) were placed in silica glass crucibles and the residual water was removed by heating over a bunsen burner. The samples were then dry-ashed at 550°C for 26 h in a muffle furnace with the lids on the crucibles. Following cooling the ashed samples were solubilized in 10 ml 70% (w/w) HNO_3. Acid washed glassware was used throughout. The calcium and magnesium concentrations of the resulting samples were measured in a Varian AA-275 atomic absorption spectrophotometer.

2.2.6/ Photoaffinity labelling of P_2G and P_2R with [^{3}H]flunitrazepam

The locust and rat membrane preparations P_2G and P_2R were photoaffinity labelled with [^{3}H]FNZP following the method of Mohler et al. (1980). Membranes were resuspended (0.75 mg protein/ml) in assay buffer (10 mM TrisHCl, pH 7.4, containing 0.15 M choline chloride, 4 mM $CaCl_2$ and 4 mM $MgCl_2$) and incubated in

the presence or absence of 100 μM diazepam at 0°C for 30 min in the dark. [^{3}H]FNZP was added (20 nM final concentration unless otherwise stated) and the membranes incubated for a further 90 min at 0°C in the dark. The suspension was then illuminated for 3 min in a Rayonet Photochemical Reactor (Southern New England Ultraviolet Co.) under 'black' light of peak wavelength 350 nm. The membranes were washed four times with assay buffer (1500 volumes) containing 10 μM diazepam. The washed, photoaffinity labelled membranes were subjected to polyacrylamide gel electrophoresis under denaturing conditions (Laemmli, 1970). The gels were either subjected to fluorography or sliced and the radioactivity of the slices measured.

2.2.7/ Polyacrylamide gel electrophoresis

Membrane fractions (P_2G & P_2R) were subjected to polyacrylamide gel electrophoresis under denaturing conditions (Laemmli, 1970). Prior to loading on either slab or disc polyacrylamide gels, samples (including standards of known relative molecular mass) were boiled for 7 min in 0.125 M TrisHCl, pH 6.8, containing 2% (w/v) sodium dodecylsulphate, 2% (w/v) β-mercaptoethanol and 0.002% (w/v) bromophenol blue. Both slab and disc gels consisted of a 10% (w/v) polyacrylamide gel with a 5% (w/v) polyacrylamide stacking gel. Disc gels (10 x 0.5 cm) were electrophoresed at 0.5 mA/gel for 30 min and then at 2 mA/gel until the bromophenol dye front

approached the bottom of the gel. Slab gel electrophoresis utilized an LKB 2001 vertical gel apparatus (gel dimensions 16 cm x 18 cm x 2 mm) in which case the samples were run into the gel at 50 V for 30 min and the gel then electrophoresed until the dye front approached the bottom of the gel. Following electrophoresis, gels were fixed and stained in 45% (v/v) methanol containing 10% (v/v) glacial acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R-250, and then destained in 30% (v/v) ethanol containing 10% (v/v) glacial acetic acid. Disc gels were analysed in a gel scanner (model 1310, Fisons) using a UA-5 absorbance monitor (Fisons) set at 580_{nm}. Slab gels were analysed by either fluorography or slicing.

2.2.8/ Fluorography of polyacrylamide gels

Following electrophoresis and subsequent staining and destaining of polyacrylamide gels, the gels were dehydrated in glacial acetic acid for 5 min. The dehydrated gels were impregnated with 20% (w/v) PPO in glacial acetic acid for 90 min, and then washed in distilled water for a minimum of 3 h. Following washing, the gels were dried down onto Whatman 3 mm filter paper for 1 h on an LKB 2003 gel drier. The dried gel was then exposed to Kodak X-Omat S X-ray film for 1-3 weeks at $-80^{o}C$, and the fluorogram then developed for 4 min in Kodak LX-24 X-ray developer (20% v/v), fixed for 5 min in Kodak FX-40 liquid fixer (20% v/v), washed

extensively and dried.

2.2.9/ Analysis of polyacrylamide gels by slicing

Following electrophoresis and subsequent staining and destaining of polyacrylamide slab gels, individual lanes of the gel were removed with a scalpel and sliced at 1.7 mm intervals using a fixed series of razor blades. The slices were placed in scintillation vials, dried, and solubilized with H_2O_2 (30% w/v) containing NH_4OH (1% v/v). Toluene scintillant (10 ml) containing 30% (v/v) Triton X-100 and 0.5% (w/v) PPO was added and the radioactivity counted.

2.2.10/ Protein determination

The protein concentration of samples was measured by the method of Lowry et al. (1951). When samples were in choline-containing buffers, a white precipitate was formed, on addition of the Folin Ciocalteu's reagent. This was removed by centrifugation at 3000 x g for 15 min in an MSE Mistral 6L centrifuge. In this case choline was introduced into the protein standards and these were subjected to the same procedure. The treatment did not affect the protein concentration values obtained and this was confirmed in parallel protein determinations with both samples and standards made up in both choline-free and choline-containing buffers.

The Biorad protein assay (Bradford, 1976) was

investigated as an alternative to the method of Lowry et al. (1951). However, although the Biorad assay was unaffected by choline chloride, it gave protein values for unknown samples which were 20% of the values obtained for the same samples by the Lowry method. Therefore to permit comparison of the results from different binding assays the modified Lowry assay described above was used routinely for protein determination.

2.3/ RESULTS

2.3.1/ The binding of [^{3}H]muscimol to the locust ganglionic membrane fraction, P_2G

The various analyses of the binding of [^{3}H]muscimol to P_2G were carried out as described in 2.2.3. In all experiments assays were carried out in triplicate.

2.3.1.1/ DEVELOPMENT OF THE [^{3}H]MUSCIMOL BINDING ASSAY

A study was made of the [^{3}H]muscimol binding activity in the different stages of the ganglion fractionation scheme. High non-specific binding was encountered in the homogenate and the P_1 pellet (low speed centrifugation, Fig. 5) making it impossible to obtain absolute values for binding activity. However the implication was that a large proportion of the original binding activity was carried through the S_1 supernatants to P_2G. In early experiments, following solubilization of the membrane pellets, 1 ml scintillant was added directly to the Eppendorf tube which was then put in a minivial for counting. The counting efficiency was increased by over 50% when the contents of the Eppendorf tube were put directly into the counting vial and 5 ml scintillant added. Transfer of the Eppendorf contents into the counting vial by either washing through with scintillant, or cutting off the bottom of the tube with

scissors, gave very variable counts. This was alleviated by removing the tube bottoms with a hot scalpel blade. Solubilization of the membrane pellet produced in the binding assay by the reagent Soluene led to as much as 10% chemiluminescence which was reduced to under 1% by neutralization of the Soluene with an equal volume of glacial acetic acid. The addition of an antiprotease cocktail (0.1 mM PMSF, 0.1 mM benzethonium chloride, 2 mM benzamidine hydrochloride) to the preparative buffers had no effect on the binding of [^{3}H]muscimol to P_2G.

2.3.1.2/ THE EFFECT OF INCREASING PROTEIN

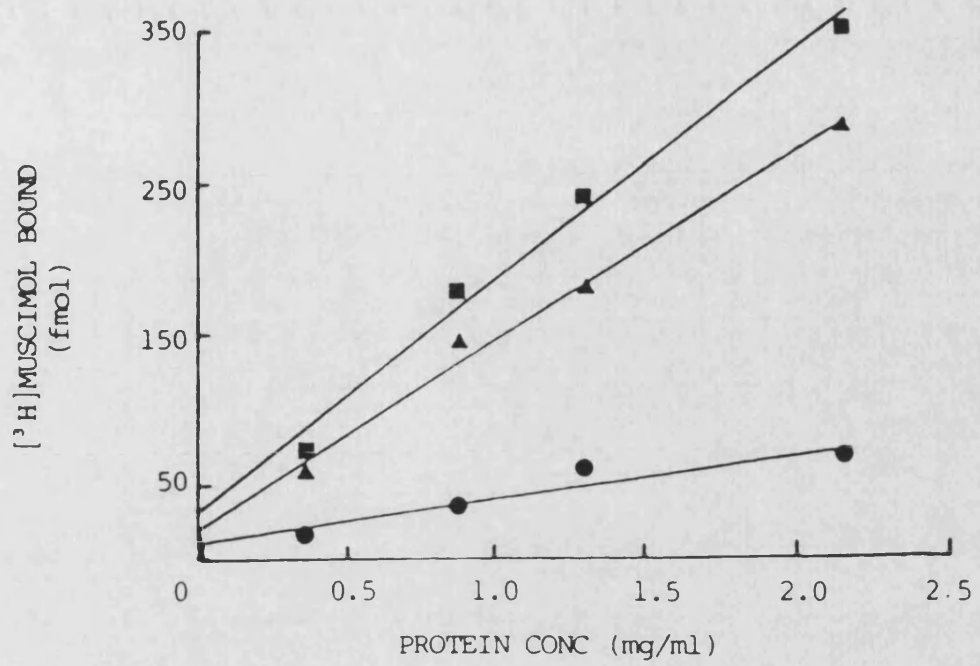
The binding of [^{3}H]muscimol (20 nM) to P_2G was measured over the protein concentration range 0.25-2.5 mg/ml. Total, non-specific and specific binding are compared in Fig.6. All subsequent experiments were performed at a protein concentration of 1 ± 0.2 mg/ml.

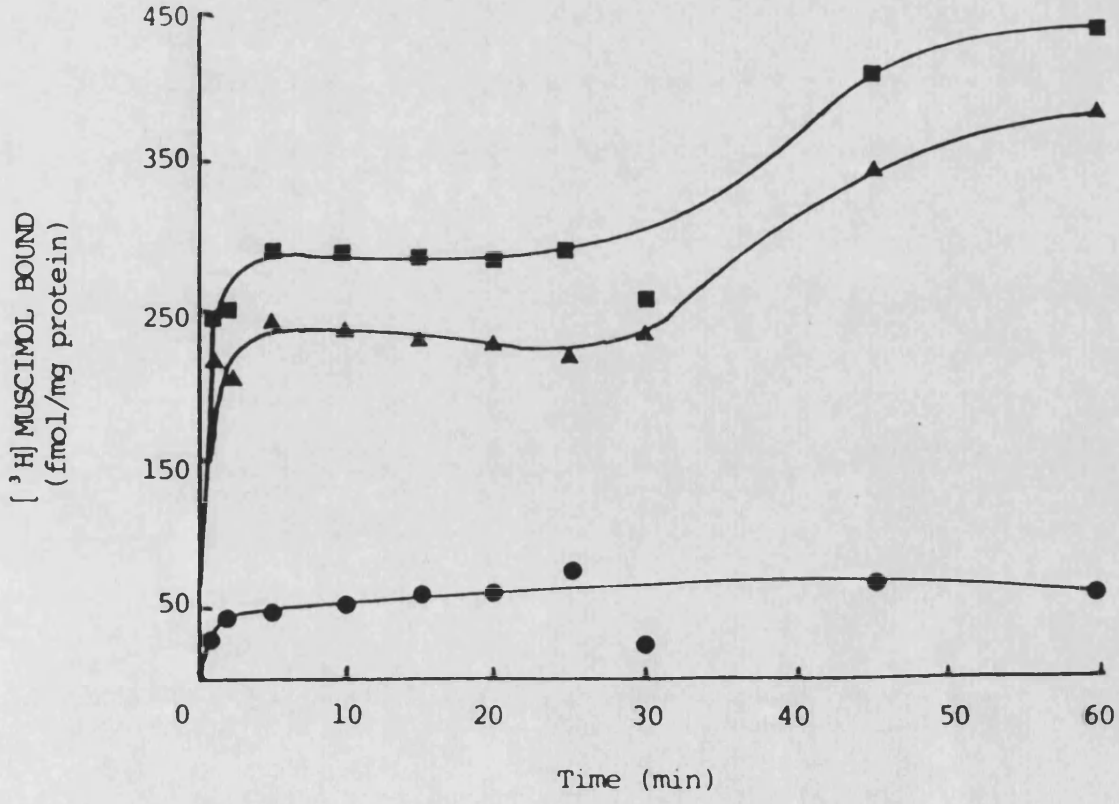
2.3.1.3/ TIME COURSE OF [^{3}H]MUSCIMOL BINDING TO P_2G

The binding of [^{3}H]muscimol (20 nM) to P_2G was measured at times up to 60 min in the presence of Na^+ ions. Total, non-specific and specific binding are compared in Fig.7. Binding saturated in 10 min and all subsequent assays employed a 30 min incubation time.

Fig.6 Effect of protein concentration on total (■), non-specific (▲) and specific (●) [^{3}H]muscimol binding (20 nM) to P_2G. Results are the means of two experiments. Assays were performed in triplicate.

Fig.7 Time course of total (■), non-specific (▲) and specific (●) binding of [^{3}H]muscimol (20 nM) to P_2G. Results are the means of two experiments. Assays were performed in triplicate.


[³H]MUSCIMOL BOUND
(fmol)
350
250
150
50
0
0.5
1.0
1.5
2.0
2.5
PROTEIN CONC (mg/ml)


[³H] MUSCIMOL BOUND
(fmol/mg protein)
450
350
250
150
50
0
10
20
30
40
50
60
Time (min)

2.3.1.4/ EFFECT OF INCREASING [^{3}H]MUSCIMOL CONCENTRATION

Specific binding of [^{3}H]muscimol to P_2G was measured over the range 2-20 nM [^{3}H]muscimol, and the resulting binding curve is shown in Fig.8a. These data were then subjected to Scatchard analysis (Scatchard, 1949) (Fig.8b) where

$$B/F = (B_{max}-B)K_D^{-1}$$

B = concentration of bound [^{3}H]muscimol

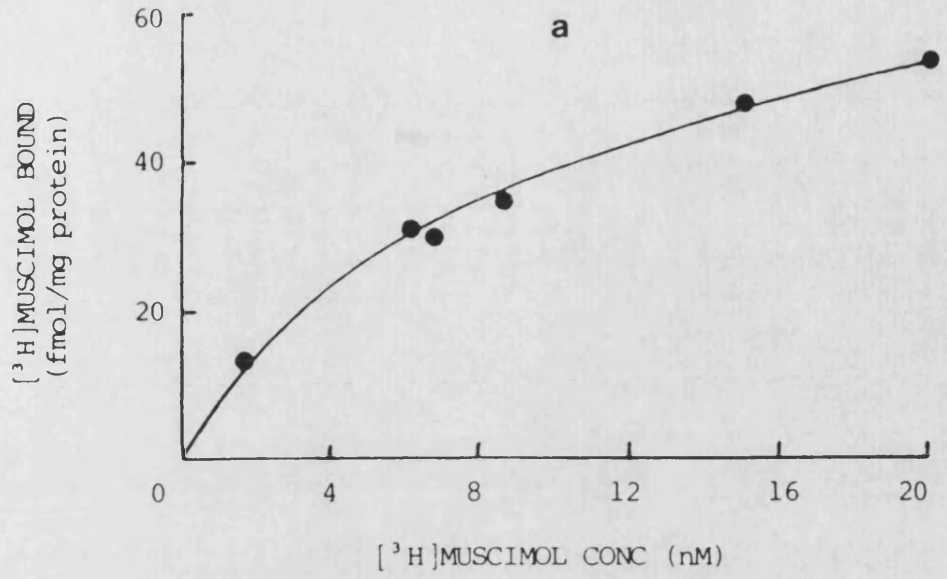
F = concentration of free [^{3}H]muscimol

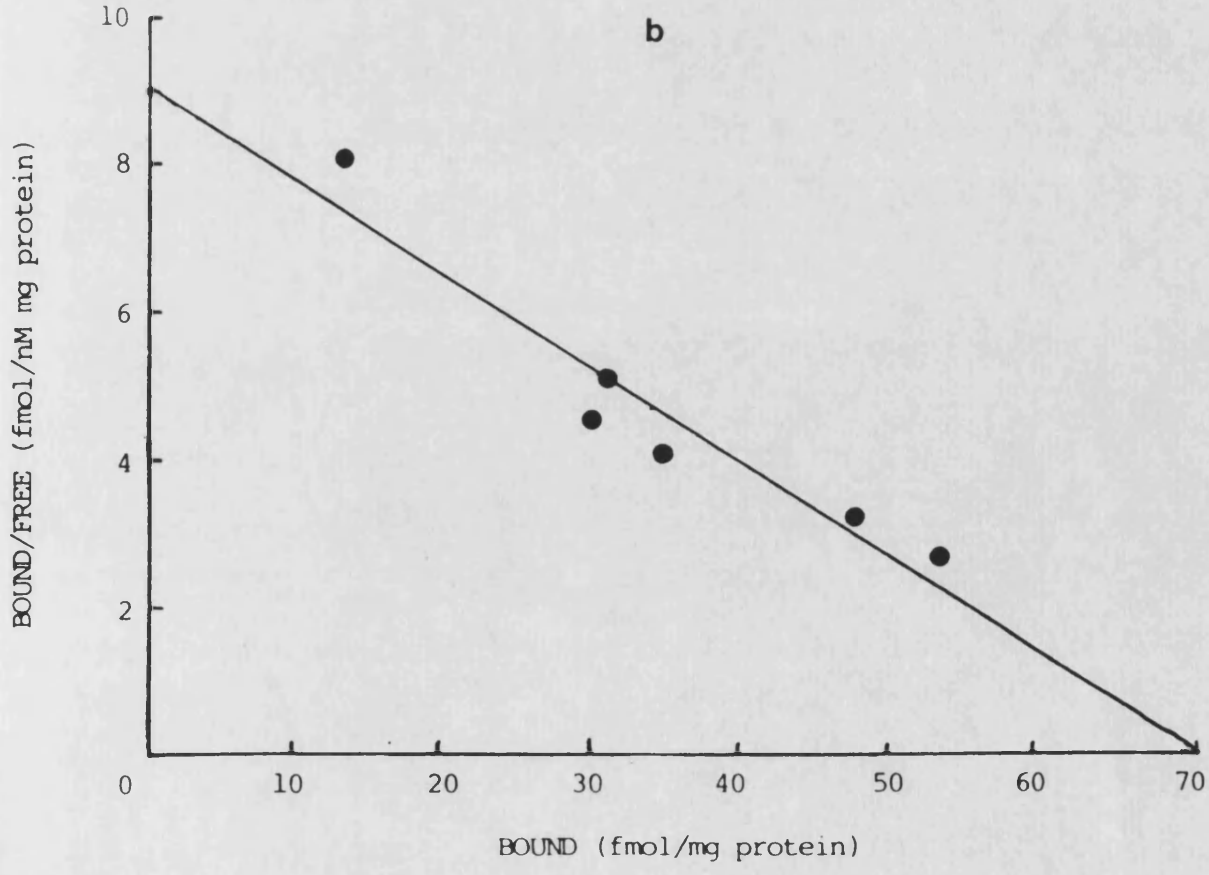
K_D = equilibrium dissociation constant

B_{max} = total concentration of [^{3}H]muscimol binding sites

The linear Scatchard plot of the ratio of bound to free [^{3}H]muscimol versus the concentration of bound [^{3}H]muscimol suggests the presence of a single class of binding sites. From this analysis (linear regression, r, = 0.95) a dissociation constant, K_D, of 8 nM, and a maximum number of [^{3}H]muscimol binding sites, B_{max}, of 70 fmol/mg protein were obtained for the binding of 2-20 nM [^{3}H]muscimol to P_2G. Routinely the non-specific binding represented 75% of the total [^{3}H]muscimol binding to P_2G.

Fig.8 a) Effect of increasing [^{3}H]muscimol concentration on specific binding of [^{3}H]muscimol to P_2G, and b) Scatchard analysis of the binding curve presented in a). The data are from a single experiment (using triplicate assays) typical of four.


a
60
40
20
0
4
8
12
16
20
[³H]MUSCIMOL BOUND
(fmol/mg protein)
[³H]MUSCIMOL CONC (nM)


b
10
8
6
4
2
0
10
20
30
40
50
60
70
BOUND/FREE (fmol/nM mg protein)
BOUND (fmol/mg protein)

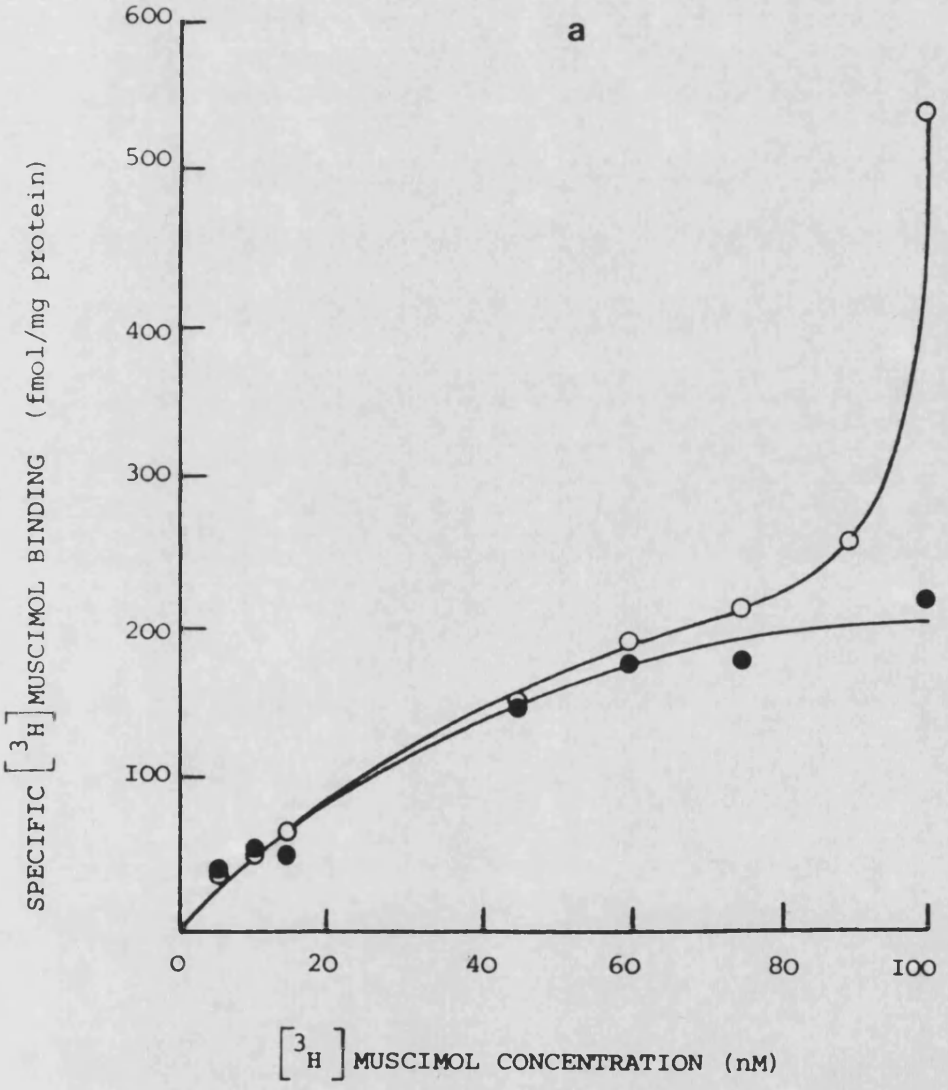
2.3.1.5/ EFFECT OF Na^+ IONS ON [^{3}H]MUSCIMOL BINDING TO P_2G

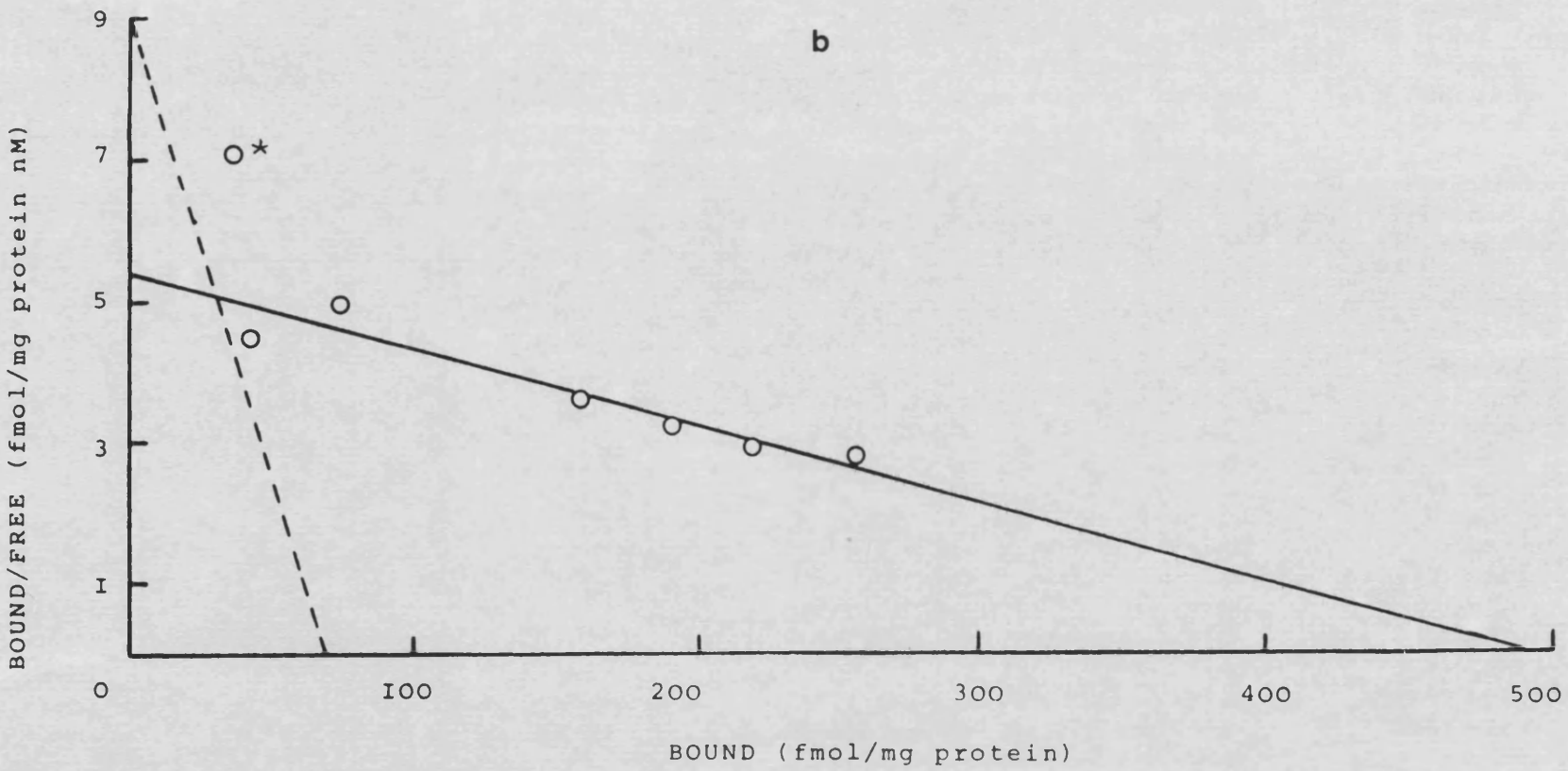
Binding of [^{3}H]muscimol to P_2G was assayed using 5-100 nM [^{3}H]muscimol in the presence and absence of Na^+ ions (152 mM). The results are compared in Fig.9. Scatchard analysis of the binding of 10-90 nM [^{3}H]muscimol (Figs.9b&c) revealed a second class of binding sites of K_D 78 nM and B_{max} 405 fmol/mg protein in the absence of sodium, and K_D 90 nM and B_{max} 498 fmol/mg protein in the presence of sodium.

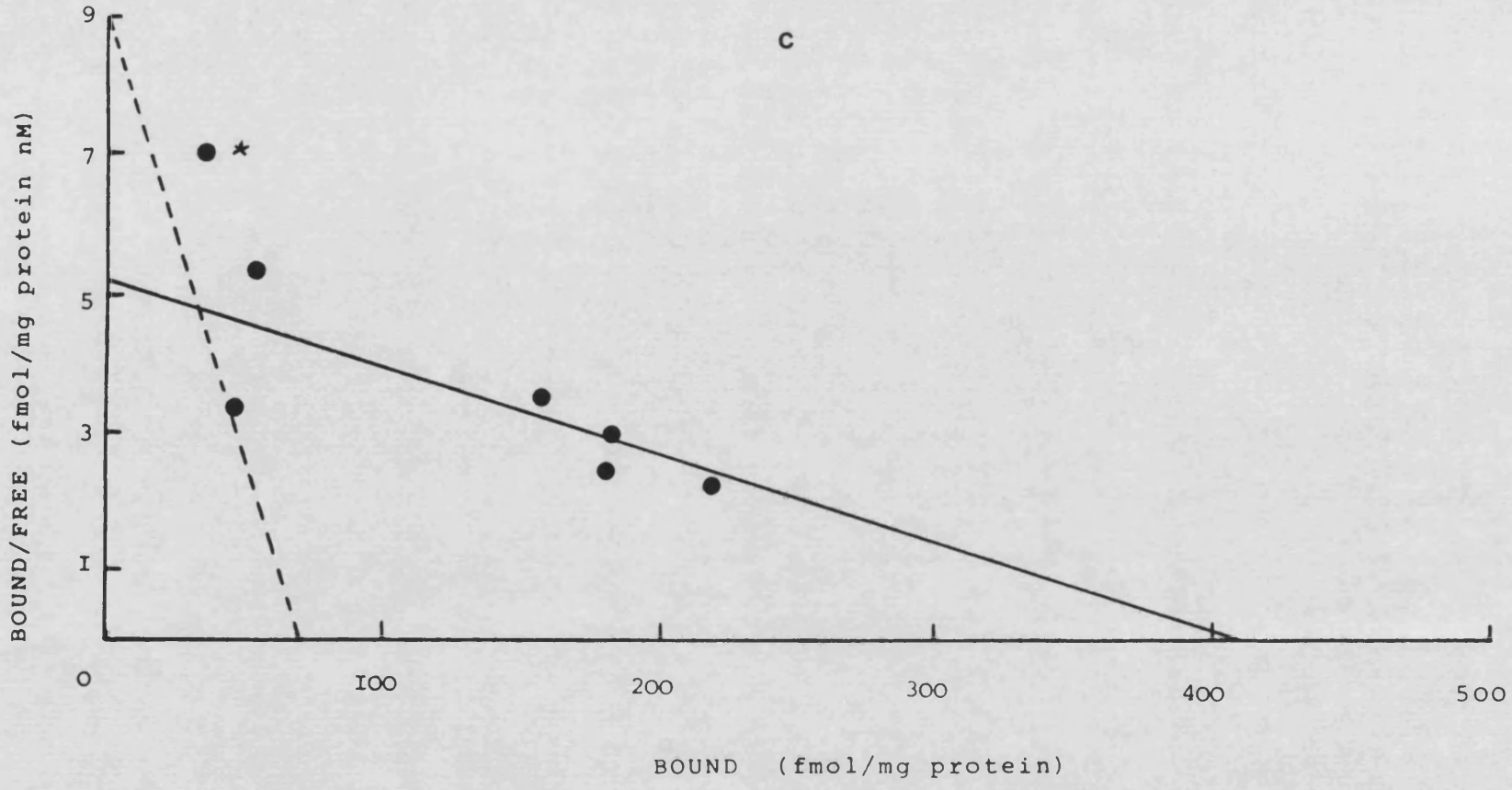
2.3.1.6/ EFFECT OF LIGANDS ON [^{3}H]MUSCIMOL BINDING TO P_2G

The effects of GABA, muscimol, isoguvacine, 3-aminopropane sulphonate, bicuculline methiodide, sodium pentobarbital and flunitrazepam on [^{3}H]muscimol binding (20 nM) were studied in P_2G. The difference in binding in the presence and absence of 10^{-3} M GABA represents 100% standard binding, and the IC_{50} is the concentration of ligand which produces 50% inhibition of that binding. The results are presented in Table 4. The effect of flunitrazepam is not presented because in the presence of Soluene this ligand produced a yellow/green colouration which interfered with the counting of radioactivity.

Fig.9 a) The specific binding of [^{3}H]muscimol to P_2G in the presence (O) and absence (●) of Na^+ ions. Results are the means of two experiments. Assays were performed in triplicate.
b) & c) Scatchard analysis of the specific binding of [^{3}H]muscimol to P_2G in the presence (b) and absence (c) of Na^+ ions. Both b) & c) are based on a linear single site analysis and do not take account of the lowest concentration point (5 nM, *). Furthermore in b) the binding at 100 nM [^{3}H]muscimol is not included. In both 9b) & c) a broken line indicates the results presented above (Fig.8b) of the Scatchard analysis of the binding of 1-20 nM [^{3}H]muscimol to P_2G in the absence of Na^+ ions.


a
600
500
400
300
200
100
0
20
40
60
80
100
SPECIFIC [3H] MUSCIMOL BINDING (fmol/mg protein)
[3H] MUSCIMOL CONCENTRATION (nM)


b
9
7
5
3
1
0
100
200
300
400
500
BOUND/FREE (fmol/mg protein nM)
BOUND (fmol/mg protein)


C
9
7
5
3
I
0
100
200
300
400
500
BOUND/FREE (fmol/mg protein nM)
BOUND (fmol/mg protein)

COMPOUND	IC_{50} (μM)
MUSCIMOL	0.03
GABA	0.1
ISOGUVACINE	0.2
3-AMINOPROPANE SULPHONATE	3.0
BICUCULLINE METHIODIDE	N.E
PENTOBARBITAL	N.E

Table 4 Effect of ligands on [^{3}H]muscimol binding in P_2G. "N.E" = No consistent effect at 1 mM concentration.

2.3.2/ Benzodiazepine binding studies on P_2G and P_2R

2.3.2.1/ DEVELOPMENT OF THE [^{3}H]FNZP BINDING ASSAY

The centrifugation assay used to measure [^{3}H]muscimol binding (2.2.3) was initially used to analyse [^{3}H]FNZP binding to the ganglionic membranes, P_2G, but the flunitrazepam interacted with the Soluene (used to solubilize the resulting membrane pellets) to give a yellow/green colour which caused elevation of the counts. Therefore the filtration assay of Braestrup & Squires (1977) described in 2.2.4 was used to investigate [^{3}H]FNZP binding to rat brain membranes.

Specific binding of [^{3}H]FNZP to P_2R was observed in the presence of EGTA, but the EGTA had to be removed to obtain specific binding of [^{3}H]FNZP to P_2G (see subsequent results, 2.3.2.2-5). All the subsequent analyses of [^{3}H]FNZP binding to P_2G and P_2R were made by the filtration assay described in 2.2.4 using assay buffer which lacked EGTA. Control experiments showed that there was no specific benzodiazepine binding to GF/C filters and ethanol (2% v/v), at a greater concentration than that used to dissolve cold ligand, did not affect background binding of [^{3}H]flunitrazepam to either filters or membranes. Presoaking of filters in 1% (w/v) lysine for 10 min prior to their use did not reduce non-specific binding.

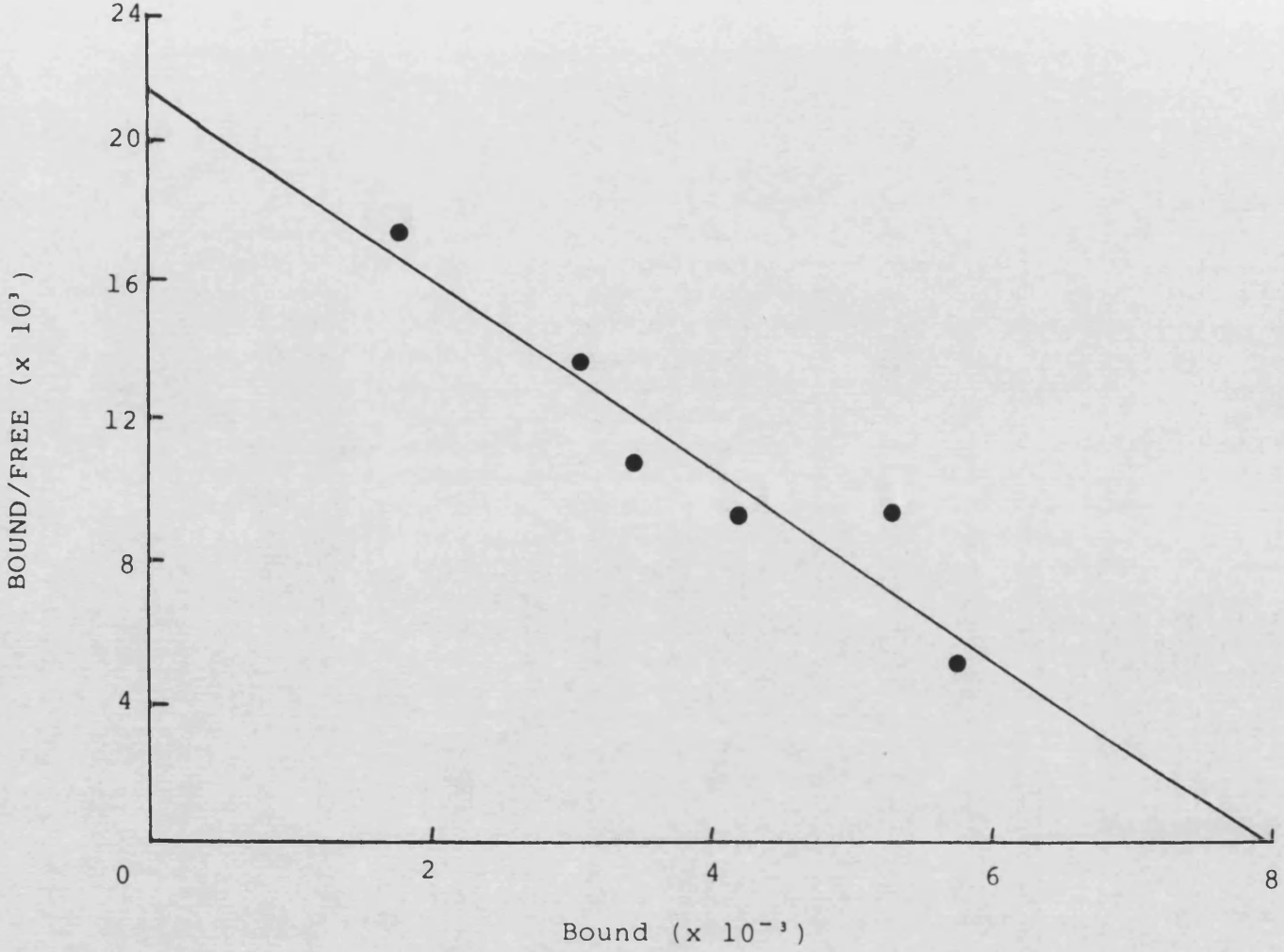
2.3.2.2/ BINDING OF [^{3}H]FNZP TO P_2R

A rat brain membrane fraction, P_2R, was prepared as described in 2.2.2. Specific binding of [^{3}H]FNZP was observed in buffers containing EGTA. Scatchard analysis of this binding (Fig.10) gave a K_D of 6.4 nM.

2.3.2.3/ EFFECTS OF Ca^{2+}, Mg^{2+}, AND EGTA ON THE BINDING OF [^{3}H]FNZP TO P_2G AND P_2R

Membrane fractions were produced from both locust ganglia (P_2G, 2.2.1) and rat brain (P_2R, 2.2.2).

Fig.10 Scatchard analysis of the binding of [^{3}H]FNZP to P_2R in the presence of EGTA. Data are from a single experiment employing triplicate assays. Similar data were obtained in a separate experiment.


24
20
16
12
8
4
0
2
4
6
8
BOUND/FREE (x 10³)
Bound (x 10⁻³)

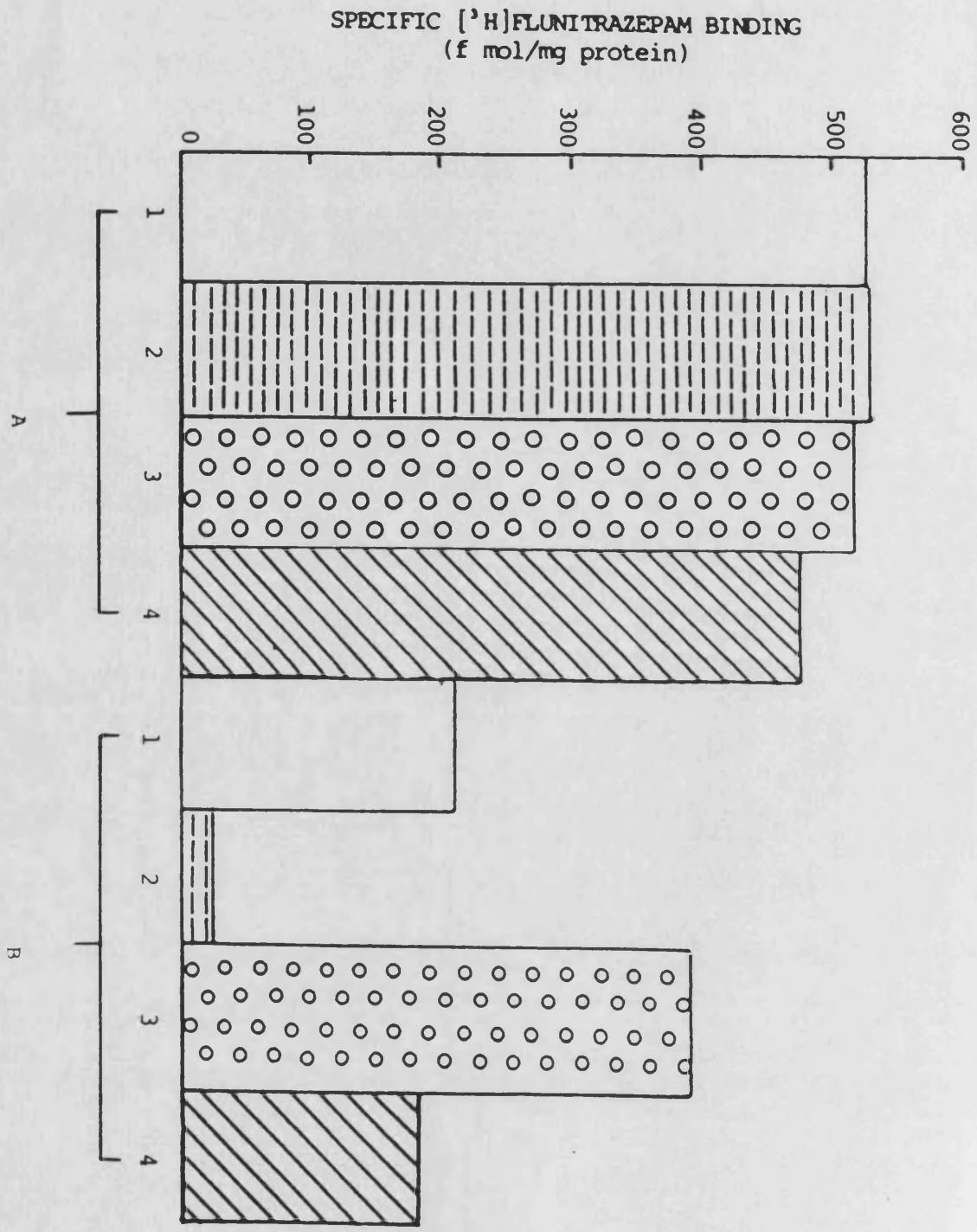
The binding of 20 nM [^{3}H]FNZP was compared in both preparations in an assay buffer of 10 mM TrisHCl, pH 7.4, containing only 0.15 M choline chloride. Specific binding in this 'blank' buffer was compared with specific binding in the same assay buffer containing 1 mM EGTA, 4 mM $CaCl_2$ or 4 mM $MgCl_2$. The results are presented in Fig.11.

Furthermore the effect of increasing concentrations of $CaCl_2$ (0-20 mM) was investigated on [^{3}H]FNZP binding to P_2G. Standard [^{3}H]FNZP binding (100%) represents the binding in assay buffer lacking Ca^{2+} (or EGTA), and was always enhanced (up to 100%) by the addition of Ca^{2+}. The Ca^{2+} concentration giving maximum enhancement was always in the 1-5 mM range, and a typical Ca^{2+}-enhancement curve from four separate experiments is presented in Fig.12.

2.3.2.4/ DETERMINATION OF TISSUE CONCENTRATIONS OF CALCIUM AND MAGNESIUM

The concentrations of calcium and magnesium in rat brain, locust ganglion and the membrane preparations, P_2R and P_2G, obtained from these tissues were determined by atomic absorption spectroscopy (2.2.5). The resulting histograms are shown in Figs. 13 and 14.

Fig.11 Effects of (1) no buffer additions, (2) 1 mM EGTA, (3) 4 mM Ca^{2+} and (4) 4 mM Mg^{2+} on the specific binding of [^{3}H]FNZP (20 nM) to P_2R (A) and P_2G (B). Results are the means of two to four experiments using triplicate assays.


SPECIFIC [³H]FLUNITRAZEPAM BINDING
(f mol/mg protein)
0
100
200
300
400
500
600
A
1
2
3
4
B
1
2
3
4

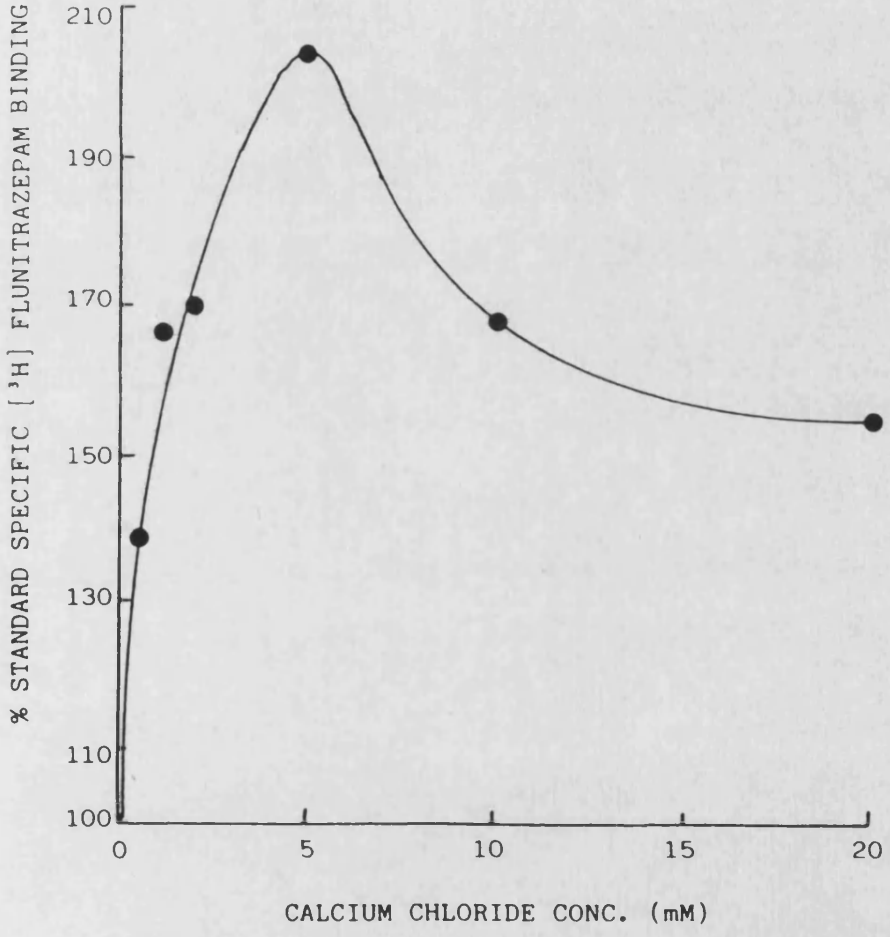


Fig.12 Effect of $CaCl_2$ concentration on [^{3}H]FNZP binding (20 nM) to P_2G. Results are from a single experiment typical of three, employing triplicate assays.

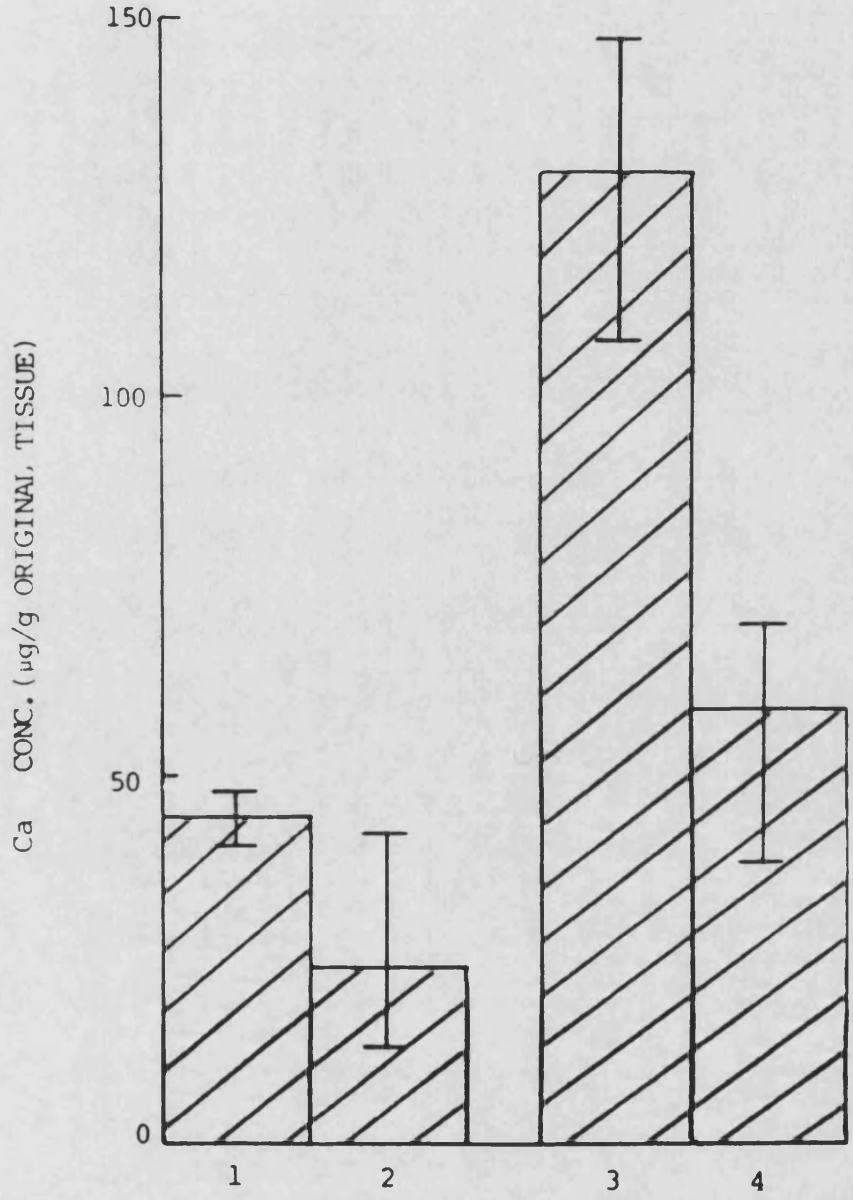


Fig.13 Concentration of calcium in rat brain (1), locust ganglion (3) and the membrane preparations P_2R (2) and P_2G (4) derived from them. The histograms show the mean results of three separate determinations. Bars denote the upper and lower values of the three results.

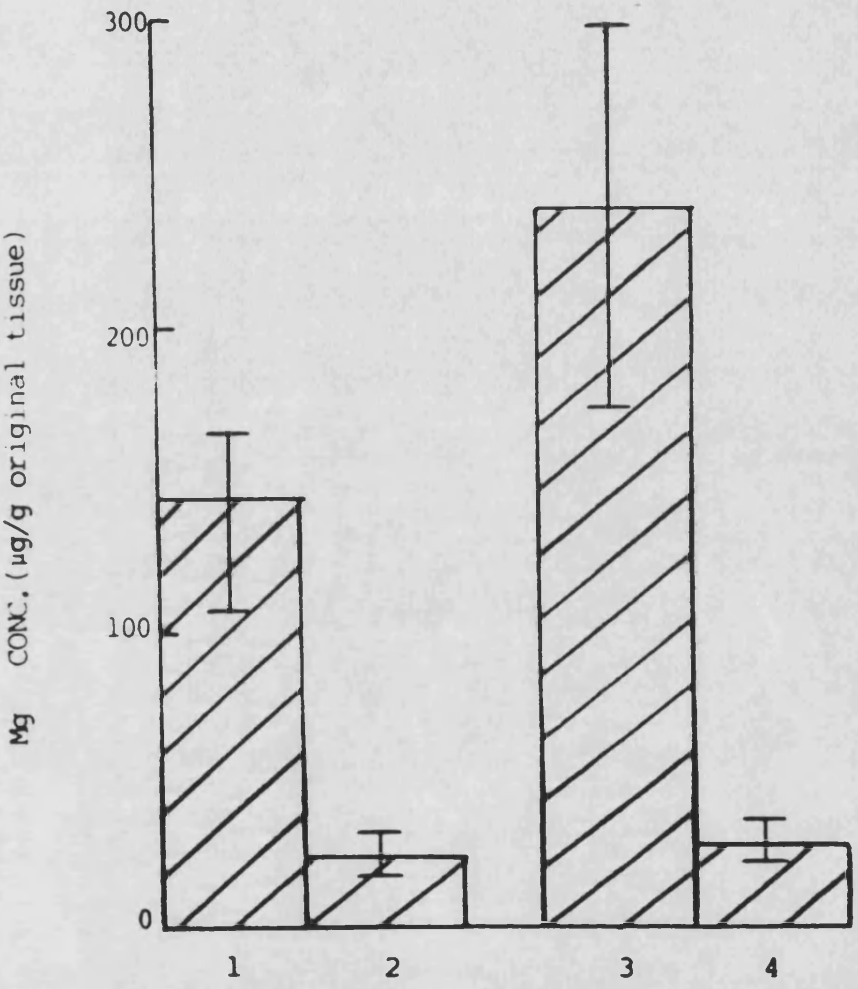


Fig.14 Concentration of magnesium in rat brain (1), locust ganglion (3) and the membrane preparations P_2R (2) and P_2G (4) derived from them. The histograms show the mean results of three separate determinations. Bars denote the upper and lower values of the three results.

2.3.2.5/ BINDING OF [^{3}H]FNZP TO P_2G

The binding of [^{3}H]FNZP to P_2G was measured by the filtration assay previously described (2.2.4). No consistent displacement of 20 nM [^{3}H]FNZP was observed with cold flunitrazepam, diazepam, clonazepam, or Ro5-4864 when assay buffer contained 1 mM EGTA. However [^{3}H]FNZP was displaced by cold diazepam when 1 mM EGTA was replaced by 4 mM $CaCl_2$ and 4 mM $MgCl_2$ in the assay buffer (see above, 2.3.2.3). All subsequent assays of [^{3}H]FNZP binding to P_2G were performed in assay buffer containing 4 mM $CaCl_2$ and 4 mM $MgCl_2$.

2.3.2.5.1/ Effect of increasing protein concentration

Binding of 15 nM [^{3}H]FNZP to P_2G was measured over the protein concentration range 0.02-0.42 mg/ml. Total, non-specific and specific binding are compared in Fig.15. Specific binding was linear with respect to protein in this range and all subsequent [^{3}H]FNZP binding assays were performed with a P_2G protein concentration of 0.2 ± 0.05 mg protein/ml.

2.3.2.5.2/ Effect of increasing [^{3}H]FNZP concentration

Specific binding of [^{3}H]FNZP to P_2G was assayed using 5-100 nM [^{3}H]FNZP and a resulting binding curve, typical of eight such experiments, is shown in

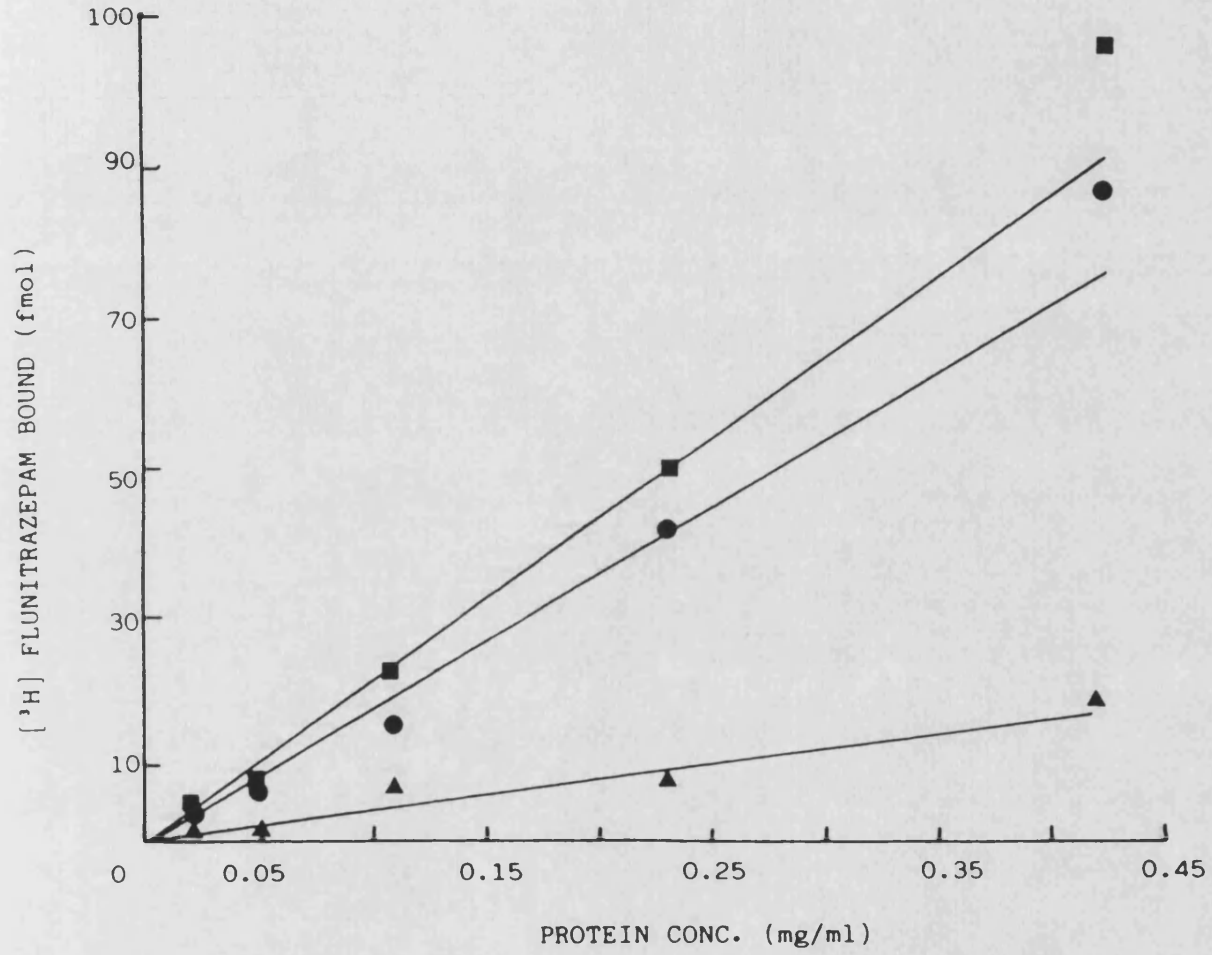


Fig.15 Effect of protein concentration on total (■), non-specific (▲) and specific (●) binding of 15 nM [^{3}H]FNZP to P_2G. Results are the mean of two experiments employing triplicate assays.

Fig.16a. These data were further analysed using the logarithmic relationship first described by Hill (1910) (Fig.16b) where

$$\log [B/(B_{max}-B)] = n_H \log F - \log K_D$$

B = concentration of bound [^{3}H]FNZP

F = concentration of free [^{3}H]FNZP

K_D = Equilibrium dissociation constant of [^{3}H]FNZP

B_{max} = Total concentration of [^{3}H]FNZP binding sites

n_H = The Hill coefficient

A plot of $\log(B/[B_{max}-B])$ versus logF, known as the Hill plot, indicates deviations of receptor/ligand interactions from the classic mass-action hyperbolic behaviour, the slope of the line giving the Hill coefficient, n_H; an n_H value of unity indicates a single population of non-interacting sites. The Hill coefficient obtained from Fig.16b is 0.91.

The binding data were also subjected to Scatchard analysis (Fig.16c). The linear Scatchard analysis of the ratio of bound to free [^{3}H]FNZP versus the concentration of bound [^{3}H]FNZP assumes the presence of a single class of binding sites. Such analyses gave K_D values in the range 17-47 nM and B_{max} values of 0.4-0.6 pmol/mg protein.

Note that the above binding characteristics were not affected by freezing of ganglia at -80^{o}C (for

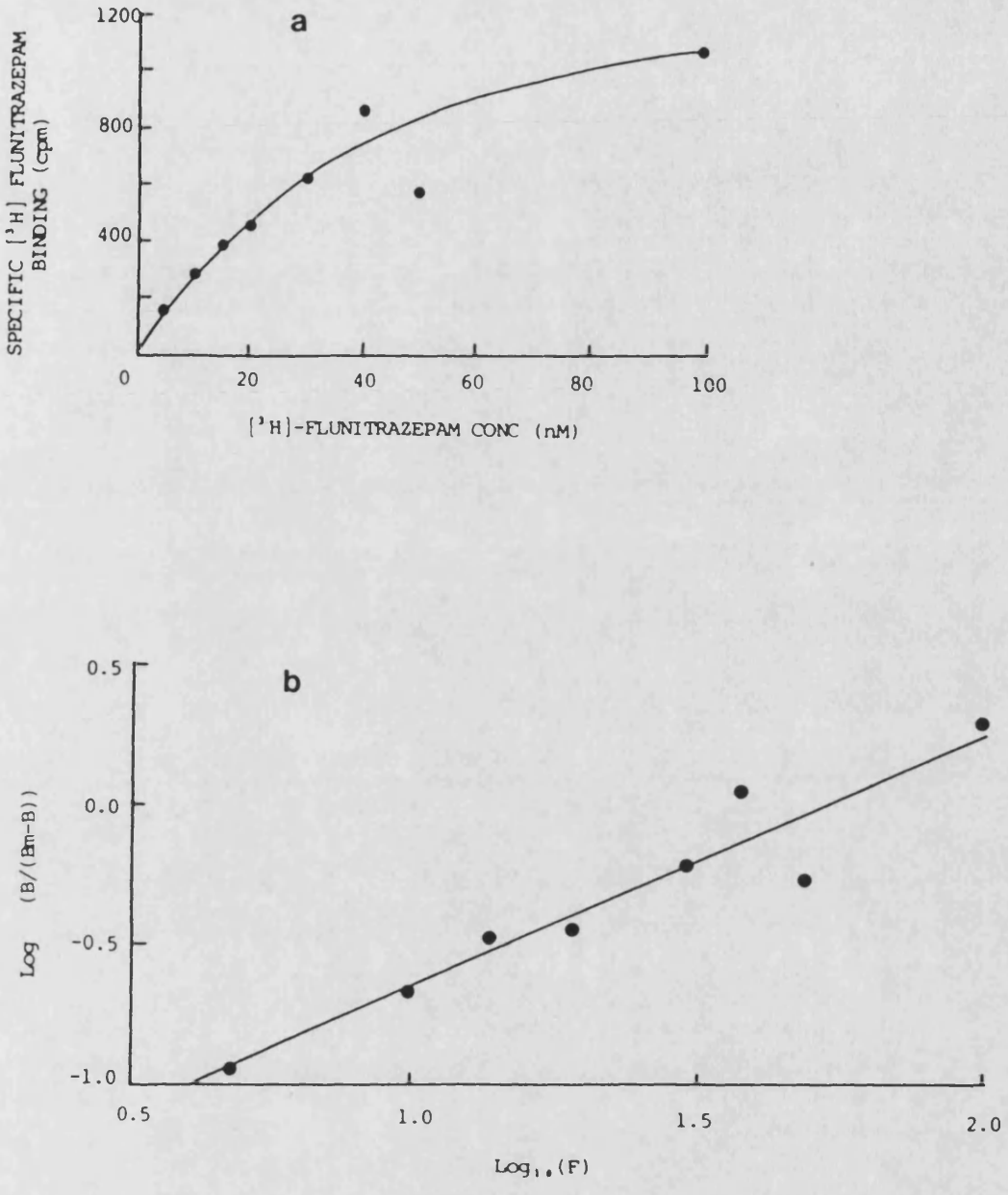
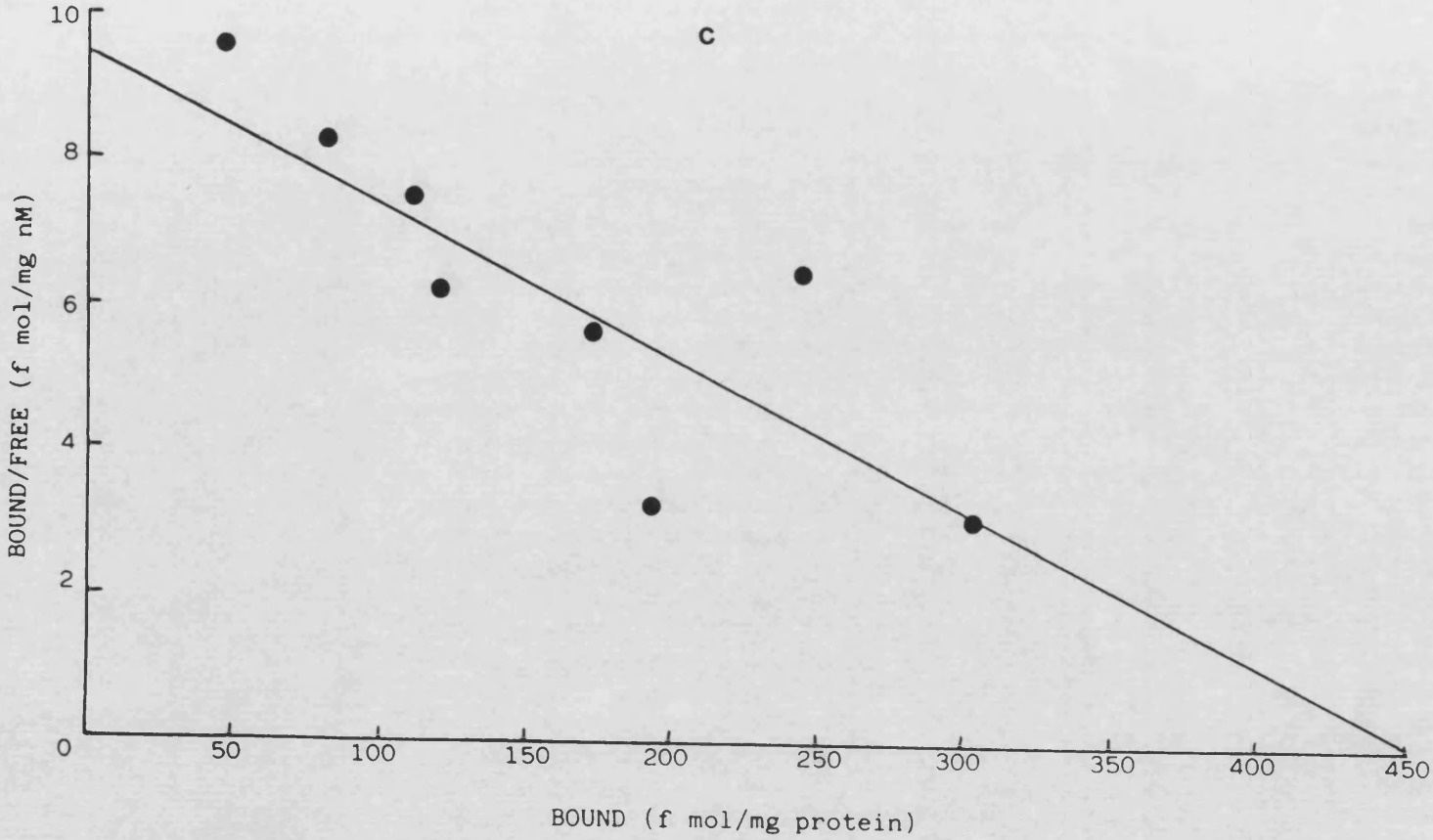


Fig.16 a) Effect of increasing [^{3}H]FNZP concentration on specific binding of [^{3}H]FNZP to P_2G.
b) Hill analysis, and c) Scatchard analysis of the binding curve presented in a). The data are from a single experiment typical of eight, using triplicate assays.


C
10
8
6
4
2
0
BOUND/FREE (f mol/mg nM)
50
100
150
200
250
300
350
400
450
BOUND (f mol/mg protein)

up to 1 month) prior to the preparation of P_2G. Routinely the specific binding represented 80% of the total [^{3}H]FNZP binding to P_2G.

2.3.2.5.3/ Determination of the rate of dissociation of [^{3}H]FNZP from P_2G

The dissociation of specifically bound [^{3}H]FNZP from P_2G was analysed as described in 2.2.4. Membranes were incubated in the presence of 15 nM [^{3}H]FNZP for 30 min, and then, following the addition of unlabelled diazepam to a final concentration of 0.1 mM, samples were filtered as described in 2.2.4 at set time points. From the resulting dissociation curve (Fig.17a) the time required for 50% dissociation of the ligand, $t_{\frac{1}{2}}$, is approximately 4 min.

The binding of a ligand, L, to a receptor, R, is described by the equation

$$R + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RL$$

and

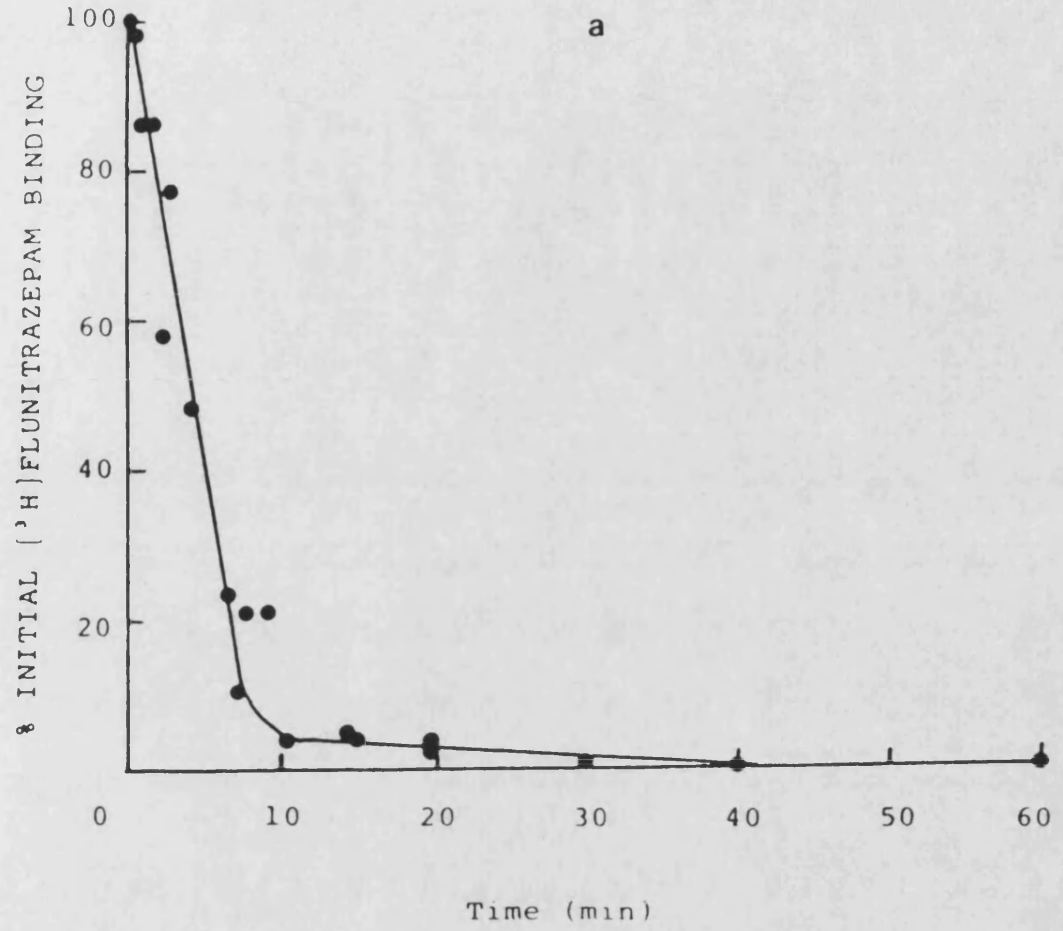
$$\ln(B/B_0) = -k_{-1}t$$

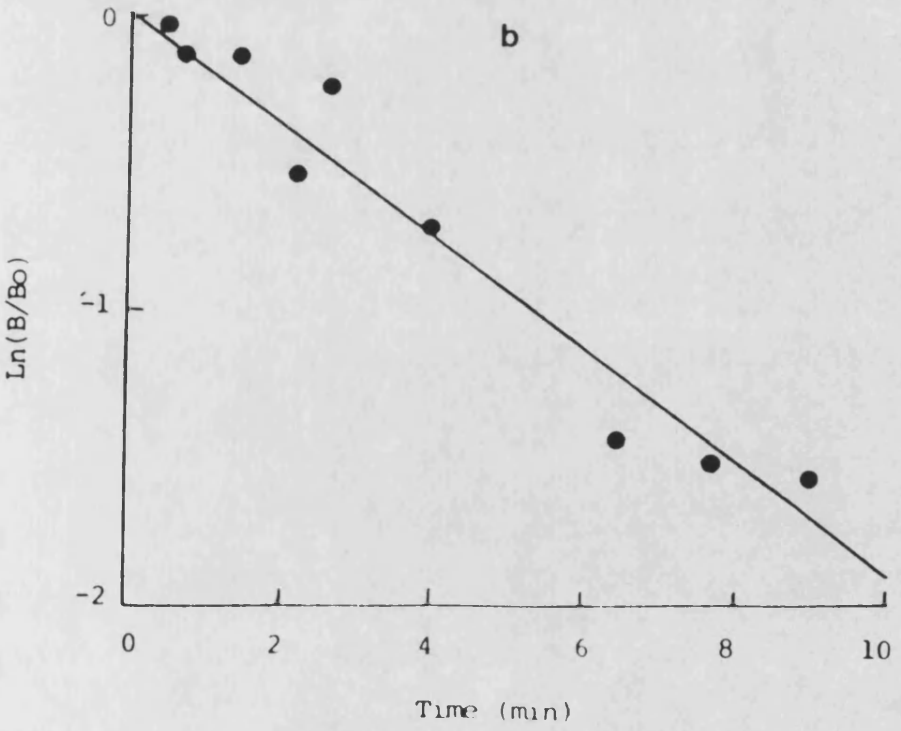
where B = [^{3}H]FNZP bound at time, t

B_0 = [^{3}H]FNZP bound at t = 0

Thus a plot of $\ln(B/B_0)$ versus time (Fig. 17b) has a slope of $-k_{-1}$. By linear regression analysis the

Fig.17 a) The dissociation of [^{3}H]FNZP (15 nM) from P_2G following infinite dilution with unlabelled diazepam, and b) the secondary plot of the dissociation curve. Results are the mean of two experiments employing triplicate assays.


a
% INITIAL [³H]FLUNITRAZEPAM BINDING
100
80
60
40
20
0
10
20
30
40
50
60
Time (min)


b
Ln(B/Bo)
0
-1
-2
0
2
4
6
8
10
Time (min)

off-rate constant, k_{-1}, for the dissociation of [^{3}H]FNZP from P_2G is 0.189min^{-1}.

2.3.2.5.4/ Determination of the rate of association of [^{3}H]FNZP with P_2G

Specific binding of [^{3}H]FNZP to P_2G was measured at time points up to 120 min and the resulting time course of association of [^{3}H]FNZP with P_2G (Fig.18a) demonstrates 90% association of the ligand in 30 min.

There are two ways in which the association rate, k^{+1}, may be calculated; these assume second order or pseudo-first order kinetics. When data are treated as second order, only the initial rate of binding is considered and thus the bound ligand concentration is assumed to be so low that no reverse reaction occurs. Pseudo-first order on the other hand includes the reverse reaction but assumes that the concentration of radioactive ligand is constant, and this treatment is preferable for receptor binding studies (Bylund, 1980). By first order treatment, as a reaction is allowed to go to equilibrium the bound concentration of [^{3}H]FNZP, B, is related to the equilibrium concentration of bound [^{3}H]FNZP, B_e, (Williams & Lefkowitz, 1978; Bennett, 1978)

thus:

$$\ln(B_e/[B_e-B]) = (k'_{+1}+k_{-1})t$$
$$= k_{ob}t$$
$$\text{where } k'_{+1} = k_{+1}L$$

where k_{ob} represents the experimentally observed apparent rate constant and is obtained by plotting $\ln(B_e/[B_e-B])$ versus time. However this analysis gave a non-linear secondary plot (Fig. 18b), possibly indicative of a heterogeneity in the binding sites. When the k_{ob} was obtained from the initial 5 min of association, and knowing k_{-1} (see above, 2.3.2.5.3), a value was obtained for k_{+1} of 0.0074 $min^{-1}nM^{-1}$, since:

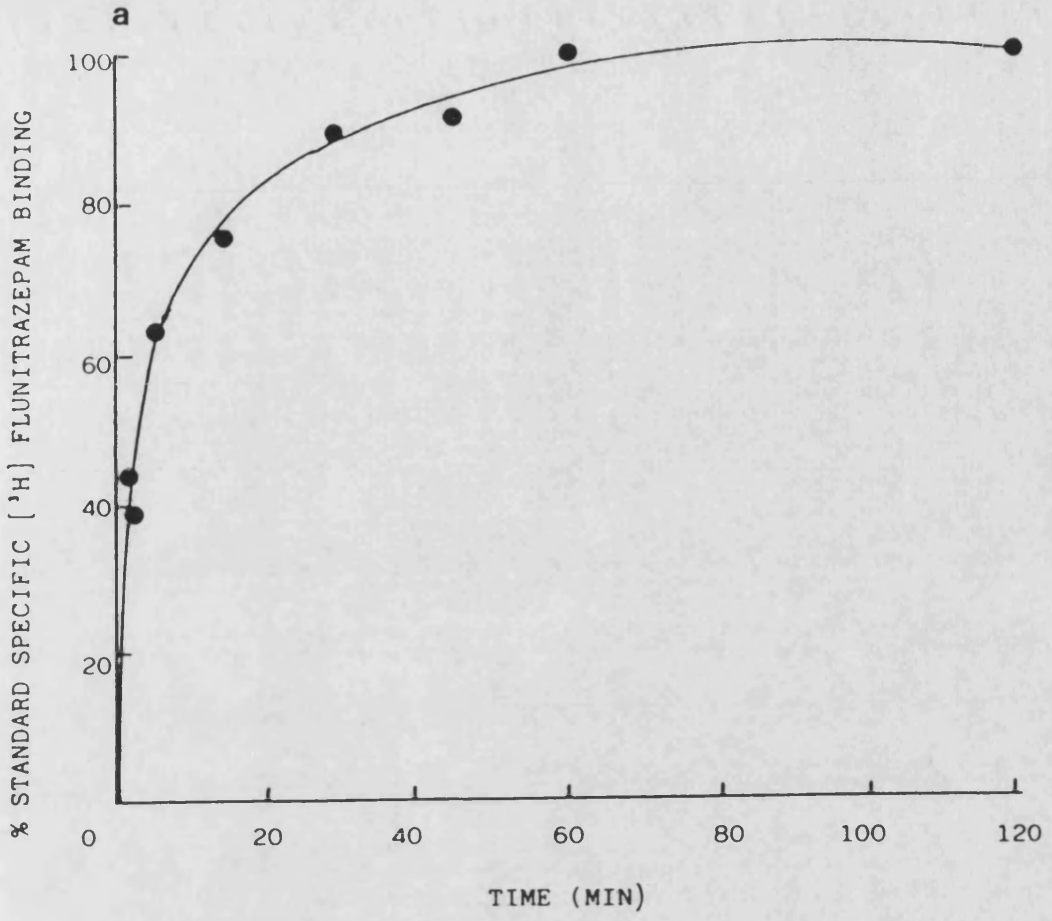
$$k_{ob} = k_{+1}L_t + k_{-1}$$

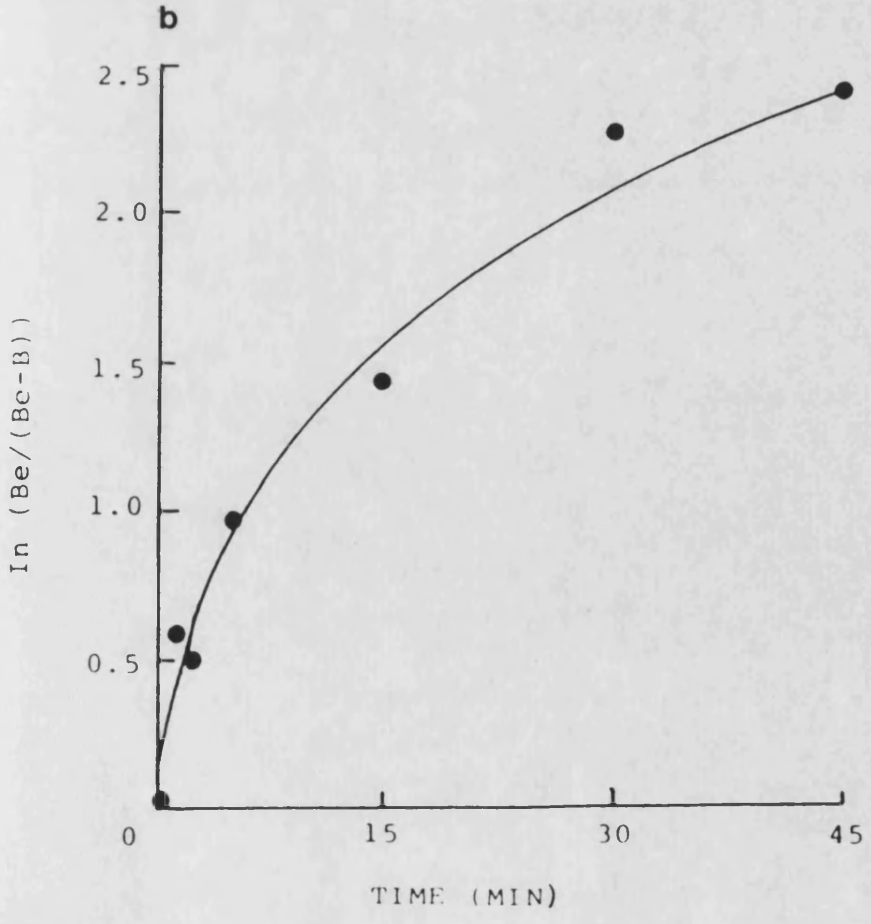
Furthermore a dissociation constant was then derived from the rate constants by the relationship

$$K_D = k_{-1}/k_{+1}$$
$$= 25.5nM$$

This value is within the range of values obtained for K_D by equilibrium binding studies (2.3.2.5.2).

Fig.18 a) The association of [^{3}H]FNZP (15 nM) with P_2G and b) the secondary plot of the association data. Results are the mean of two experiments employing triplicate assays.


a
% STANDARD SPECIFIC [³H] FLUNITRAZEPAM BINDING
100
80
60
40
20
0
20
40
60
80
100
120
TIME (MIN)


b
In (Be/(Bc-B))
2.5
2.0
1.5
1.0
0.5
0
15
30
45
TIME (MIN)

2.3.2.5.5/ Pharmacology of the binding of [^{3}H]FNZP to P_2G

The efficacies of a range of benzodiazepines in displacing 5 nM [^{3}H]FNZP from P_2G are illustrated in Fig.19. The ligands Ro5-4864, diazepam and flunitrazepam were of approximately equal efficacy in displacing [^{3}H]FNZP binding (IC_{50}~0.1 μM), being over 100-fold more potent than the enantiomers Ro11-5231 and Ro11-5073, and over 500-fold more potent than clonazepam. Hill plot analysis was used to obtain the Hill coefficient values (n_H) of these displacement curves; the Hill plot of displacement of [^{3}H]FNZP by flunitrazepam is shown in Fig.20. Hill coefficient values were obtained for displacement of [^{3}H]FNZP by diazepam, flunitrazepam and Ro5-4864 (Table 5), and all three indicated a heterogeneity of binding sites, having n_H values markedly less than unity.

Furthermore 0.1 mM GABA and isoguvacine both enhanced [^{3}H]FNZP binding to P_2G (Fig.21). In two separate experiments (S.E. of triplicates < 3%), 5 x 10^{-6}-10^{-8} M muscimol enhanced (up to 20%) the binding of 5 nM [^{3}H]FNZP (means significantly greater than controls by student's t-test, p=0.05), but 10^{-4} M muscimol was without effect. Inconsistent enhancement (up to 20%) of 5 nM [^{3}H]FNZP binding was observed in the presence of 10^{-5}-10^{-8} M GABA. In early experiments enhancement of the binding of 15 nM [^{3}H]FNZP by GABA was observed in a

Fig.19 Displacement of [^{3}H]FNZP binding (5 nM) to P_2G by a range of benzodiazepines:

▲- Ro5-4864

O- diazepam

●- flunitrazepam

△- Rol5-2538

□- Roll-5231

■- Roll-5073

✱- clonazepam

Results are the means of two to four experiments using triplicate assays.

% STANDARD SPECIFIC [³H] FLUNITRAZEPAM BINDING
0
20
40
60
80
100
120
-11
-10
-9
-8
-7
-6
-5
-4
LOG10 (LIGAND CONC.) (M)

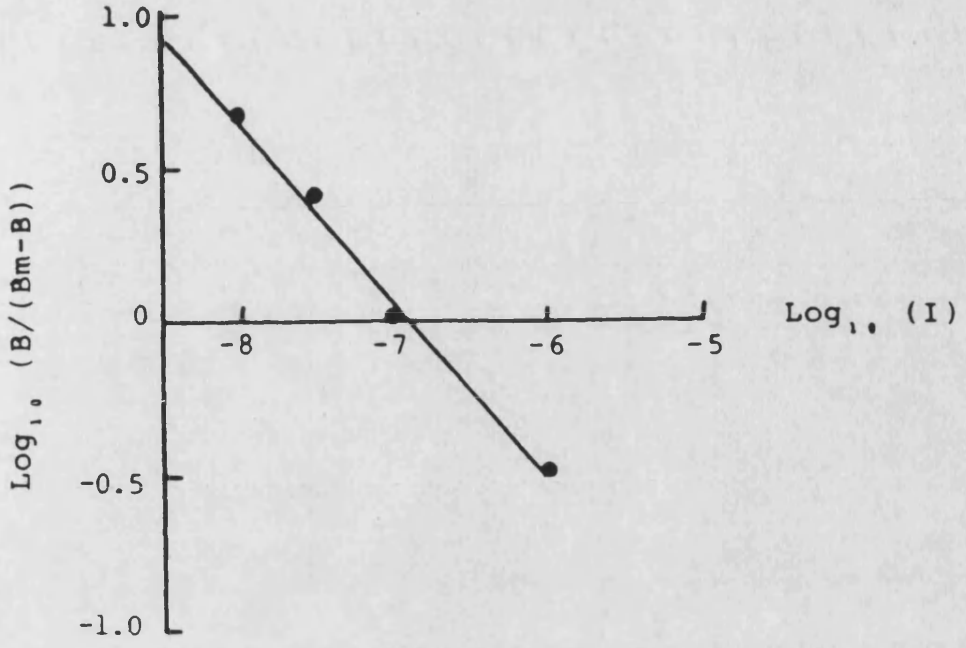


Fig.20 Hill analysis of the displacement of [^{3}H]FNZP (5 nM) from P_2G by flunitrazepam. Results are the means of three experiments using triplicate assays.

LIGAND	n_H
FLUNITRAZEPAM	0.58
Ro5 - 4864	0.64
DIAZEPAM	0.70

Table 5 Hill coefficients (n_H) for the displacement of [^{3}H]FNZP (5 nM) from P_2G by flunitrazepam, Ro5-4864 and diazepam, derived as in Fig.20.

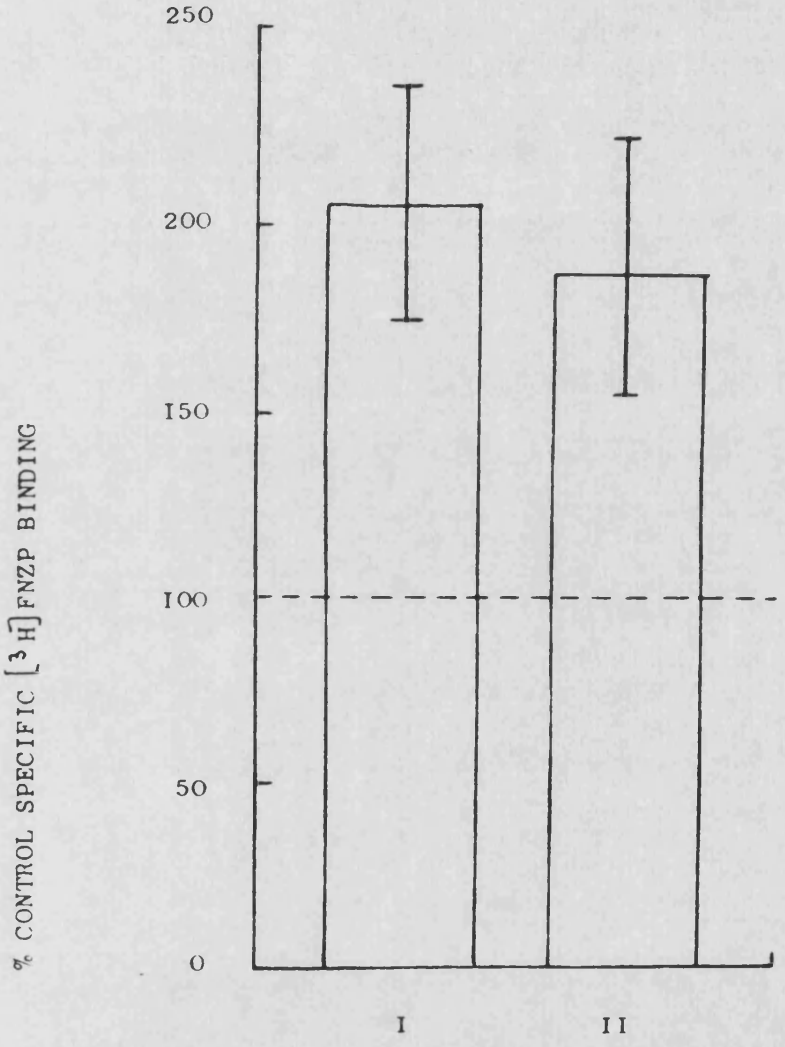


Fig.21 Enhancement of [^{3}H]FNZP binding (5 nM) to P_2G by I, 0.1 mM GABA and II, 0.1 mM isoguvacine. Results are the means of three experiments using triplicate assays, and the upper and lower values of the three results are denoted by the bars.

dose-dependent manner but this effect was not reproducible.

Effects on [^{3}H]FNZP binding in P_2G of the barbiturate, sodium pentobarbital, and of picrotoxinin were also investigated. However there was no significant effect on the binding by either ligand (at 0.1 mM or 1 mM) either under standard binding conditions or when using 22°C or 30°C incubations, Tris-malate buffer, pH 6.8, or preincubation of the membranes with the ligands prior to the addition of [^{3}H]FNZP.

2.3.3/ Photoaffinity labelling studies on P_2G and P_2R with [^{3}H]FNZP

The membrane fractions P_2G and P_2R were photoaffinity labelled with 20 nM [^{3}H]FNZP, electrophoresed on denaturing polyacrylamide slab gels (Fig.22), and the resulting gels analysed either by fluorography or slicing of the gel and counting of the radioactivity of the resulting slices (2.2.6-9).

P_2G and P_2R were photoaffinity labelled in parallel in the absence and presence of 10^{-4} M diazepam. From the resulting fluorographs of four such experiments (Fig.23) it was apparent that a single protein of M_r 48.5K was specifically labelled in P_2R whereas P_2G showed two major labelled bands, M_r 45.5±1.5K and 57±2K, and two intermediate proteins, M_r 48.5±0.5K and 53.5±1.5K were also labelled to a lesser degree.

Furthermore gels of photoaffinity labelled P_2G were analysed by gel slicing (Fig.24). In four such

experiments two specific peaks of radioactivity were always obtained, corresponding to proteins of M_r 46±0.7K and 59.5±2K and two further peaks were always obtained corresponding to proteins of M_r 50±1K and 53.5±1.5K. P_2G membranes were electrophoresed on disc gels, stained with Coomassie Blue and scanned at A_{580nm}. Such a scan, when compared with the pattern of radioactivity obtained by photoaffinity labelling of P_2G in the presence of 10^{-4} M diazepam (Fig.25), demonstrates that this non-specific labelling reflects very closely the protein profile of the membrane.

GABA, at a concentration of 10^{-8} M, enhanced the specific photoaffinity labelling of P_2G by 15 nM [^{3}H]FNZP in a single experiment (Fig.26). However, 10^{-4} M and 10^{-8} M GABA both failed to enhance the photoaffinity labelling of P_2G with 20 nM [^{3}H]FNZP in two separate experiments. Furthermore 10^{-4} M GABA failed to enhance the labelling of P_2G with 10 nM [^{3}H]FNZP in two separate experiments.

Fig.22 SDS-Polyacrylamide gel (10% w/v) of locust ganglionic P_2G membranes (4 lanes) with standards of molecular weights as indicated in the right hand lane.

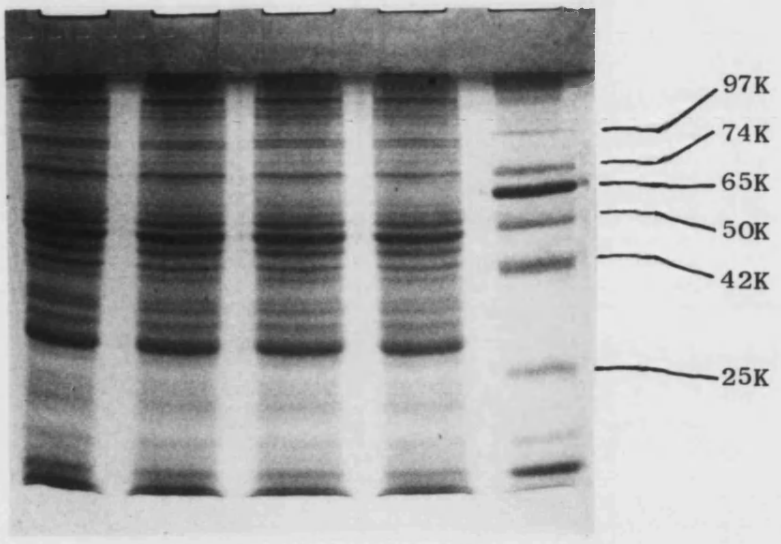
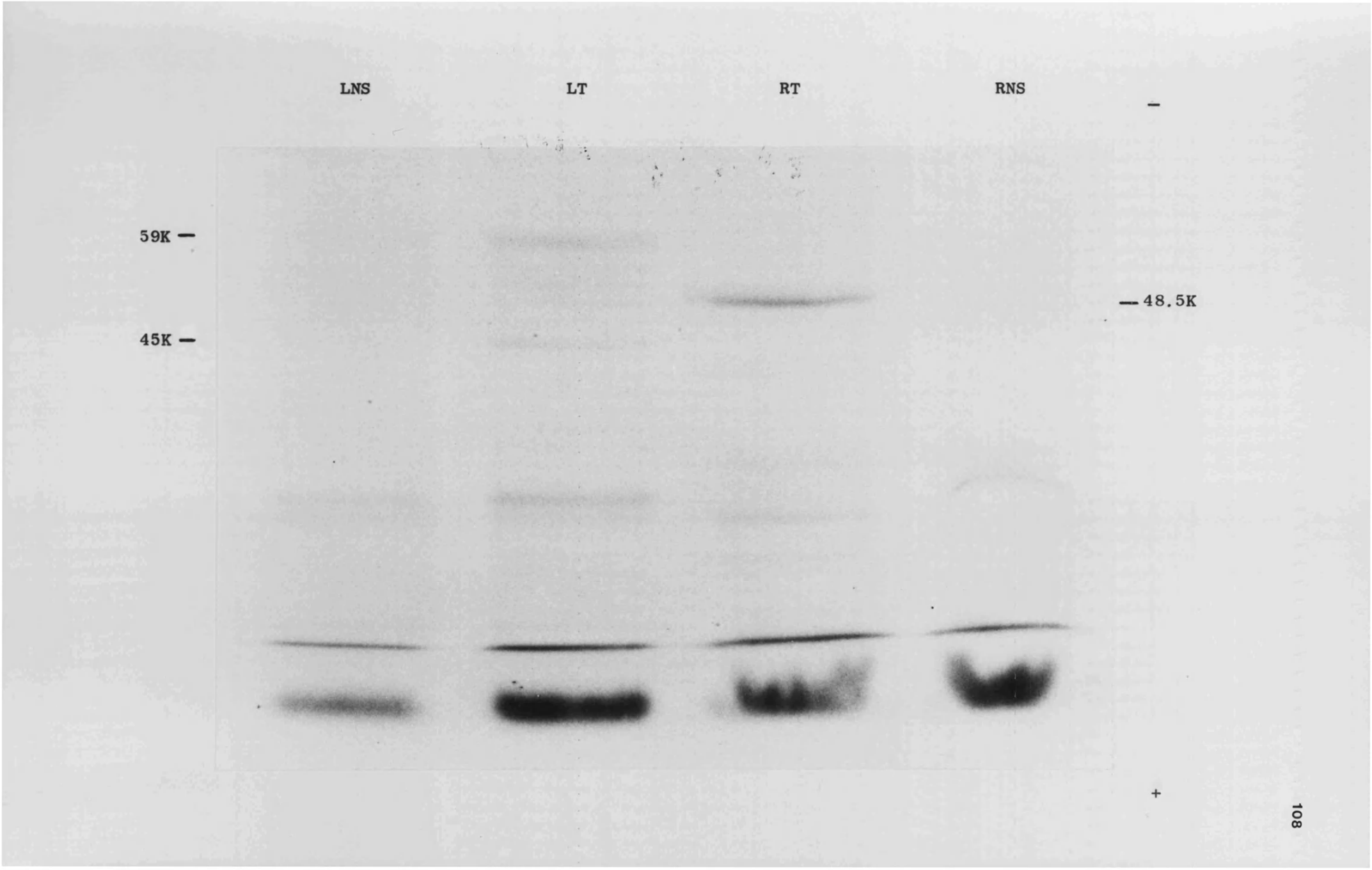

97K
74K
65K
50K
42K
25K

Fig.23 Fluorograph of photoaffinity labelled membranes from locust ganglia (L) and rat brain (R). Membranes were photoaffinity labelled with 20 nM [^{3}H]FNZP in the presence (NS) and absence (T) of 0.1 mM unlabelled diazepam. The molecular weights of the major specifically labelled bands are as indicated. The fluorograph was exposed for 10 days, and similar results were obtained in fluorographs from four separate experiments.


LNS
LT
RT
RNS
59K
45K
48.5K
108

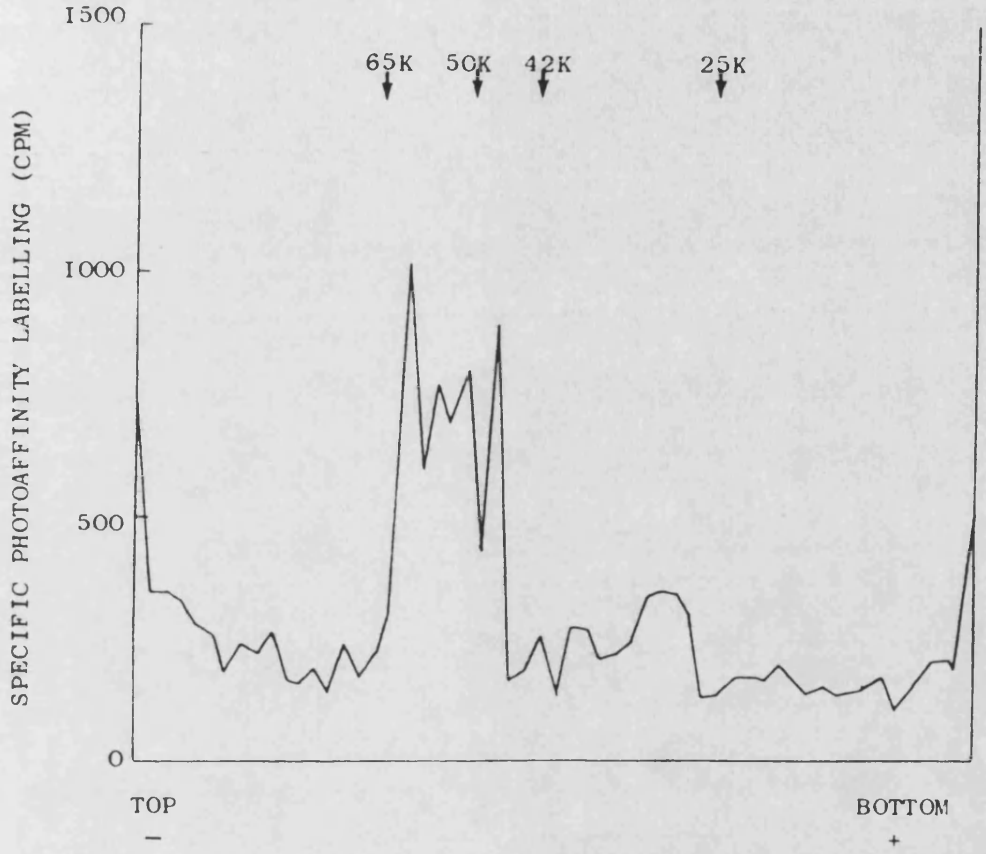
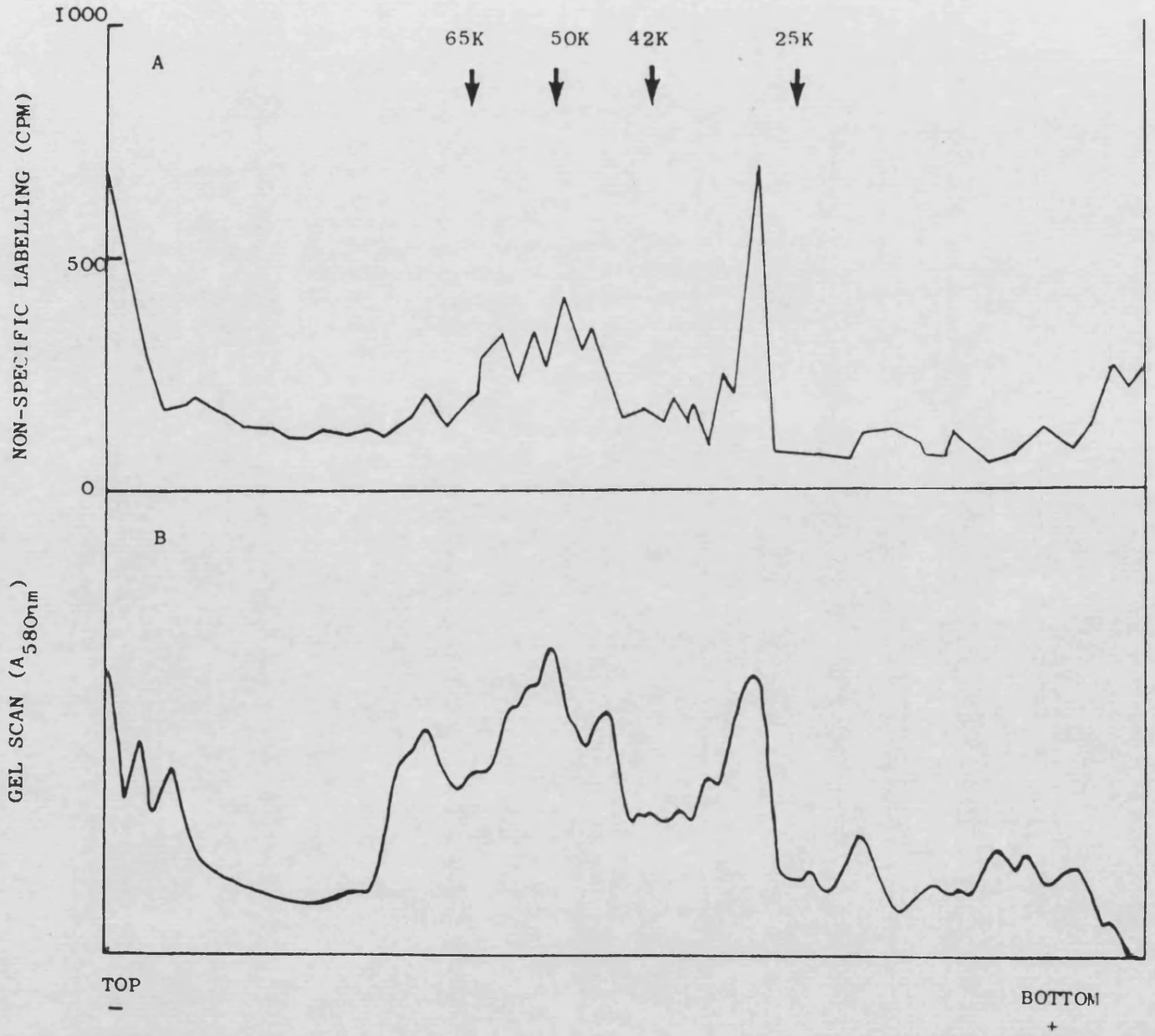


Fig.24 Slice profile of specific [^{3}H]FNZP (10 nM) photoaffinity labelling of P_2G. The profile was obtained by subtracting the radioactivity obtained by labelling in the presence from that obtained in the absence of 0.1 mM diazepam. The positions of the molecular weight standards are as indicated. Similar results were obtained in slice profiles from four separate experiments.

Fig.25 Comparison of the slice profile obtained on photoaffinity labelling P_2G with [^{3}H]FNZP (20 nM) in the presence of 0.1 mM diazepam (non-specific labelling) (A), with the protein profile of a disc gel of P_2G membranes stained with Coomassie Blue and scanned at A_{580nm} (B).


1000
500
0
A
65K
50K
42K
25K
NON-SPECIFIC LABELLING (CPM)
B
GEL SCAN (A_{580nm})
TOP
−
BOTTOM
+

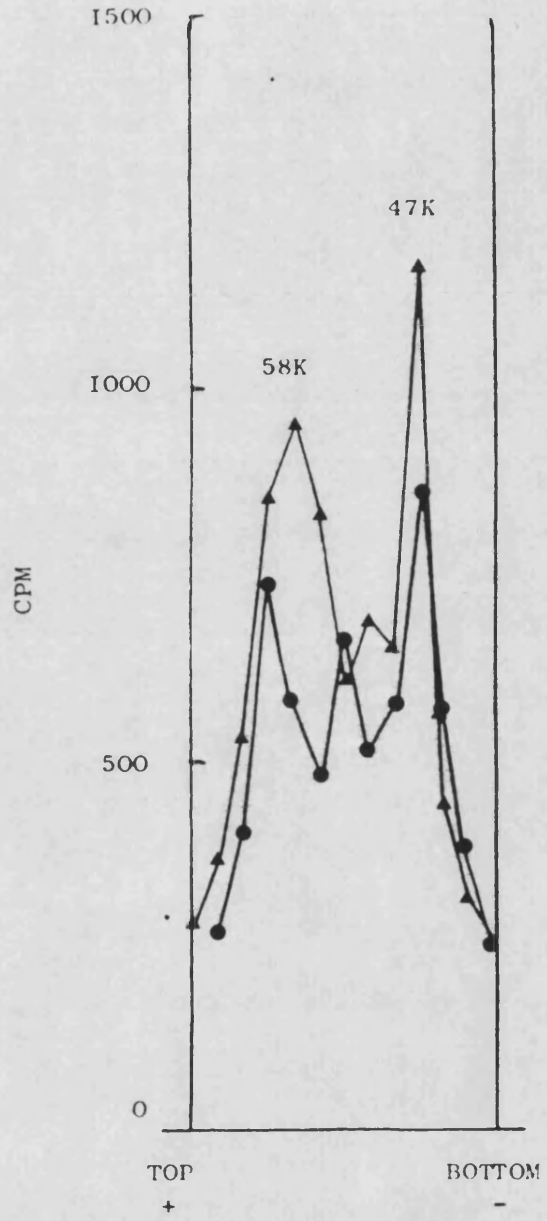


Fig.26 Enhancement of the labelling of the two major peptides (molecular weights as indicated) specifically photoaffinity labelled with 15 nM [^{3}H]FNZP (●), when labelled in the presence of 10 nM GABA (▲). Results were obtained by gel slicing and subtracting the profile obtained in the presence of 0.1 mM diazepam. They are the results of a single experiment.

2.4/ DISCUSSION

2.4.1/ The binding of [^{3}H]muscimol to the ganglionic membrane preparation, P_2G

An assay has been developed which enables the measurement of specific [^{3}H]muscimol binding to locust ganglionic membranes. The ability to detect specific [^{3}H]muscimol binding probably relies to a large extent on the extensive washing of the membranes prior to assay since mammalian brain (Greenlee et al., 1978a) and crayfish muscle (Meiners et al., 1979) contain endogenous substances which appear to inhibit muscimol binding to unwashed membranes. Muscimol will act as a substrate for GABA-T from mammalian brain (Fowler et al., 1983), but it is unlikely that the enzyme interferes with the locust binding assay as it should have been washed from the membranes during their preparation. The assay of [^{3}H]muscimol binding to P_2G was terminated by centrifugation of the membranes from the incubation medium, which led to high apparent non-specific binding of [^{3}H]muscimol. However the possibility of a rapid dissociation rate of [^{3}H]muscimol from P_2G dictated that centrifugation be used rather than filtration as a means of terminating the assay. Indeed we now know that 63% of [^{3}H]muscimol specifically bound to flyhead membranes dissociates in 1 min (Lunt et al., 1985). It is evident from the time-course data

(Fig.7) that there is no further increase in specific binding of [^{3}H]muscimol after the standard 30 min incubation time. Indeed a shorter incubation time could be used without loss of specific [^{3}H]muscimol binding. The standard assay of P_2G [^{3}H]muscimol binding was performed at an average membrane protein concentration of 0.5±0.2 mg/ml. From Fig.6 (Effect of protein concentration on [^{3}H]muscimol binding in P_2G) it is clear that specific [^{3}H]muscimol binding is linear with respect to protein concentration over this range.

In addition to sodium-independent binding of GABA, it has been reported that mammalian brain also exhibits sodium-dependent uptake of GABA (Enna & Snyder, 1975). This uptake is postulated to be a glial/neuronal removal mechanism for synaptic GABA, and it is known that such uptake occurs in insect CNS (Beadle et al., 1985; Breer & Heilgenberg, 1986; this thesis, 3.3.5). Although the [^{3}H]muscimol binding assay was carried out at 0°C which should limit a temperature-dependent process such as uptake, the binding was compared in the presence and absence of Na^+ ions (Fig.9). The data show very similar binding curves over the range 1-60 nM [^{3}H]muscimol, irrespective of the presence of Na^+ ions. However, when the [^{3}H]muscimol concentration range was extended to 100 nM, non-saturating Na^+-dependent binding was observed. Furthermore in the time-course experiments (Fig.7) when carried out at 0°C, 20 nM [^{3}H]muscimol, and in the presence of Na^+ ions, an increase in non-specific

binding is observed after 30-45 min incubation time. Whether these phenomena represent binding, uptake or adsorption of the ligand onto the membrane is unclear. Conductance increases evoked by GABA and muscimol in single lobster muscle fibres have been reported to be reduced by replacement of Na^+ ions by choline in the medium; only muscimol-evoked responses were reduced by replacement of Na^+ with Li^+ or glucosamine (Constanti & Nistri, 1981). An explanation of these data is that there are two populations of inhibitory amino acid receptors on lobster muscle, one preferring muscimol (modulated by Na^+), the other preferring GABA (less affected by Na^+ removal). This is not unreasonable as De Feudis (1980) has suggested that specific binding of muscimol in some particulate fractions of mammalian brain might not be exclusively to specific GABA binding sites. However, Beadle et al. (1985) have reported that 1 mM GABA and muscimol both cause the desensitization of cultured insect neurones and that cross-desensitization is also evident, implying that these ligands act on a common receptor population in insect CNS. Another possible explanation given by Constanti & Nistri (1981) for their data is that Na^+ can affect differentially the affinity of the same receptor site for different GABA agonists. Therefore one cannot rule out that at higher [^{3}H]muscimol concentrations Na^+ ions do affect receptor binding. However, in mammalian brain the GABA uptake system will also transport muscimol, though with 50-fold

lower affinity than for GABA (Johnson et al., 1978). It is therefore possible that a similar situation can arise in locust central nervous tissue such that at higher muscimol concentrations GABA uptake systems contribute to the measured binding of [^{3}H]muscimol. This would result in an elevated B_{max} value for the binding of 10-90 nM [^{3}H]muscimol to P_2G in the presence of Na^+ ions compared with the binding in the absence of Na^+ ions (Fig.9). Nipecotic acid and 2,4-diaminobutyrate are considered to be specific for the GABA uptake site rather than the receptor in mammalian brain (Olsen, 1981) and these compounds displayed IC_{50} values of greater than 100 μM in displacing [^{3}H]muscimol binding from housefly head membranes under conditions identical to those reported here (Lunt et al., 1985). Therefore it is concluded that GABA uptake systems do not contribute to the observed binding of 1-60 nM [^{3}H]muscimol.

The binding of [^{3}H]muscimol to locust ganglionic membranes is saturable, exhibiting a K_D of 10 nM based on a single site Scatchard analysis of the binding of 1-20 nM [^{3}H]muscimol (Fig.8b). This K_D value is comparable with the values quoted for [^{3}H]muscimol binding in mammalian brain of 5.1 nM in rat (Williams & Risley, 1979), 9 nM in mouse (Wang et al., 1979) and 3 nM in cow (Greenlee & Olsen., 1979), and with the values of 9 nM quoted for crayfish muscle (Meiners et al., 1979) and 12 nm for the purified $GABA_A$ receptor complex from bovine brain (Sigel et al., 1983). When the

concentration of [^{3}H]muscimol was extended, Scatchard analysis (Fig.9c) of the Na^+-independent binding of 10-90 nM [^{3}H]muscimol to P_2G demonstrated the presence of a second binding site of K_D 80 nM and B_{max} 400 fmol/mg protein. The existence of two binding sites of high and low affinity is comparable with the multiple affinities of mammalian $GABA_A$ receptor binding and indeed it is thought that it is the lower affinity state of the GABA receptor which is physiologically relevant (Olsen et al., 1981). Abalis & Eldefrawi (1986) have also reported two affinities for [^{3}H]muscimol binding in honeybee brain.

The availability of tissue limited studies on the pharmacology of [^{3}H]muscimol binding to P_2G. However a small range of ligands was tested for any inhibitory effect on [^{3}H]muscimol binding (Table 4). Recently there has been an increase in interest in invertebrate CNS GABA receptors and several groups have probed insect CNS with both [^{3}H]muscimol and [^{3}H]GABA (Lunt et al., 1985; Lummis & Sattelle, 1985a; Breer & Heilgenberg, 1986; Tanaka & Matsumura, 1985; Abalis & Eldefrawi, 1986). A common denominator in nearly all these studies is a virtual lack of any sensitivity of insect CNS GABA receptors to bicuculline. The characteristics of various invertebrate binding studies are compared in Table 6 with the data from a selection of mammalian GABAergic binding studies and with the data presented in this thesis.

Table 6 Comparison of GABAergic binding studies in invertebrate and vertebrate nervous tissues

REF.: T.T - This thesis
I - Lunt et al. (I985)
2 - Jeffery (personal communication)
3 - Abalis & Eldefrawi (I986)
4 - Lummis & Sattelle (I985a)
5 - Breer & Heigenberg (I986) NS denotes IO% displacement of binding, maximum concentration of ligand not given
6 - Meiners et al. (I979)
7 - Williams & Risley (I979)
8 - Enna & Snyder (I975)
9 - Greenlee et al. (I978b)

REF.	T.T	1	2	3	4	5	6	7	8	9
K_D(nM)	10 & 80	40	30	3 & 144	384	160	9	5 & 30	370	100
B_{max} (f mol/mg protein)	70 & 400	20	200	50 & 500	1420	2200	500	500 & 900	680	-
LIGAND (^{3}H)	MUSCIMOL	MUSCIMOL	GABA	MUSCIMOL	GABA	GABA	MUSCIMOL	MUSCIMOL	GABA	GABA
TISSUE	LOCUST GANGLIA	HOUSE FLY HEAD	LOCUST GANGLIA	HONEYBEE BRAIN	COCKROACH CNS	LOCUST GANGLIA	CRAYFISH MUSCLE	RAT BRAIN	RAT BRAIN	RAT BRAIN
IC_{50}(M)										
MUSCIMOL	3×10^{-8}	3×10^{-8}	1×10^{-7}	6×10^{-9}	7×10^{-7}	9×10^{-4}	2×10^{-8}	1×10^{-8}	-	4×10^{-8}
GABA	1×10^{-7}	4×10^{-8}	9×10^{-8}	4×10^{-8}	1×10^{-7}	-	2×10^{-7}	4×10^{-8}	4×10^{-7}	2×10^{-7}
ISOGUVACINE	2×10^{-7}	1×10^{-7}	5×10^{-5}	-	5×10^{-4}	-	6×10^{-8}	-	-	2×10^{-7}
3APS	3×10^{-6}	3×10^{-7}	-	3×10^{-5}	4×10^{-4}	NS	3×10^{-6}	3×10^{-8}	3×10^{-7}	6×10^{-7}
IMIDAZOLE ACETATE	-	6×10^{-7}	-	1×10^{-6}	-	-	2×10^{-7}	2×10^{-7}	2×10^{-7}	9×10^{-7}
BICUCULLINE	$>10^{-3}$	4×10^{-4}	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	NS	3×10^{-3}	6×10^{-6}	4×10^{-6}	4×10^{-6}
CHLORPROMAZINE	-	-	-	-	-	1×10^{-7}	-	-	2×10^{-4}	-
IMIPRAMINE	-	-	-	-	-	1×10^{-7}	-	-	-	-
2, 4 - DABA	-	$>10^{-3}$	-	$>10^{-3}$	-	NS	$>10^{-3}$	$>10^{-5}$	$>10^{-3}$	$>10^{-4}$
NIPECOTIC ACID	-	$>10^{-4}$	$>10^{-3}$	$>10^{-4}$	$>10^{-3}$	-	$>10^{-3}$	$>10^{-5}$	-	$>10^{-4}$
B-ALANINE	-	-	$>10^{-3}$	5×10^{-6}	$>10^{-3}$	-	-	$>10^{-5}$	8×10^{-5}	4×10^{-5}

The lack of bicuculline sensitivity in invertebrate GABA binding studies compared with the mammalian CNS is in agreement with Beadle et al. (1985) who reported that 10^{-5} M bicuculline failed to block GABA-evoked conductance increases measured in current- and voltage-clamped cultured locust neurones. However, apart from agreement on the lack of bicuculline-sensitivity, some of the invertebrate binding studies are conflicting on both the density of binding sites and their pharmacologies. All the studies except that of Breer & Heilgenberg (1986) are in agreement with respect to the potencies of GABA and muscimol irrespective of the radioactive ligand used, and this characteristic is similar to that of the mammalian $GABA_A$ receptor. Also Beadle et al. (1985) have suggested that muscimol and GABA act at a common population of receptors in cultured cockroach neurones. The binding of [^{3}H]muscimol in locust and housefly CNSs (this thesis; Lunt et al., 1985) and crayfish muscle (Meiners et al., 1979) is also similar to the mammalian GABA receptor in being sensitive to isoguvacine, 3-APS and imidazole acetate whereas [^{3}H]GABA binding in cockroach CNS (Lummis & Sattelle, 1985a) is relatively insensitive to either isoguvacine or 3-APS. A further discrepancy lies in the 10-fold higher number of binding sites observed when using [^{3}H]GABA compared with [^{3}H]muscimol. There is a danger (Olsen et al., 1975; Meiners et al., 1979) that when working with [^{3}H]GABA

the observed binding can be contaminated by a contribution from GABA uptake sites, a problem probably not encountered with nanomolar concentrations of [^{3}H]muscimol. Although the [^{3}H]GABA binding activity of locust CNS (Breer & Heilgenberg, 1986) was insensitive to the neuronal GABA uptake inhibitor, DABA, it was also insensitive to muscimol and strongly inhibited by the uptake inhibitors chlorpromazine and imipramine. These binding studies were carried out in Na^+-free medium, but the membranes did not undergo the same extensive washing and freeze/thaw cycles as those utilized by Jeffery (personal communication). Therefore it is possible that the membrane preparation contains sufficient residual Na^+ ions to permit GABA uptake into vesiculated membranes or [^{3}H]GABA can bind to the recognition site of its transporter in the presence of low concentrations of Na^+. To a lesser extent GABA uptake may contribute to the data of Lummis & Sattelle (1985a) as their observed [^{3}H]GABA binding is decreased by freezing, washing or detergent treatment of the membrane preparation prior to assay; these treatments elevate mammalian GABA receptor binding activity by removal of endogenous GABA (Greenlea et al., 1978a). Therefore it is suggested that GABA uptake sites may make a significant contribution to the high levels of low affinity [^{3}H]GABA binding observed by Breer & Heilgenberg (1986) and, to a lesser extent, to the binding observed by Lummis & Sattelle (1985a). This would explain the discrepancies between the

pharmacologies of the binding sites reported in these two studies and those reported for [^{3}H]muscimol binding sites (this thesis; Lunt et al, 1985; Abalis & Eldefrawi, 1986) and for [^{3}H]GABA binding sites on extensively washed membranes (Jeffery, personal communication).

As discussed above, the data in Table 6 do not agree on the level of GABA receptor binding activity in insect CNS. There is evidence from studies on cholinergic receptors that insect receptors are more labile than their mammalian counterparts (Filbin et al., 1983; Aguilar & Lunt, 1984). This coupled with small amounts of tissue may, in many cases, preclude meaningful measurements of receptor activity. If the [^{3}H]muscimol binding studies on P_2G were continued, the use of a membrane preparation which has undergone extensive freeze/thaw and wash cycles (Jeffery, personal communication) may result in an increase in binding by removal of vesiculated, endogenous GABA. Also Fisher et al. (1986) have reported that 0.05% (v/v) TritonX-100 is more efficient than several osmotic shock/wash cycles in removing endogenous GABA from mammalian brain membranes, and such treatment may result in enhanced binding of [^{3}H]muscimol to P_2G. Furthermore, as will be discussed later, the binding of [^{3}H]FNZP to the same P_2G preparation is elevated in the presence of Ca^{2+} ions. However, the assay of [^{3}H]muscimol binding to P_2G in the presence of Ca^{2+} rather than EGTA, though giving more

consistent binding, does not give any significant increase in the amount of binding (Riley, personal communication). In conclusion, from the considerations of GABA uptake outlined above it seems likely that the concentration of GABA receptors in insect CNS is no greater than 200 fmol/mg protein, such that insect CNS may well possess significantly lower densities of GABA receptors than mammalian brain, where the density of high affinity sites is in excess of 500 fmol/mg protein (Enna & Snyder, 1975; Williams & Risley, 1979).

Locust ganglionic membranes exhibit significant displaceable binding of the GABA receptor agonist, [^{3}H]muscimol. This binding is saturable, linear with respect to protein concentration, and shows a pharmacological profile which is consistent with a physiological receptor (Birdsall & Hulme, 1976). This receptor is very similar to the corresponding receptor on arthropod muscle, but differs in its pharmacology from the mammalian $GABA_A$ receptor. An obvious progression from these observations is to ask the question do these central insect GABA receptors possess associated receptors for benzodiazepines as described above for mammalian $GABA_A$ receptors? The question has two components: firstly, does insect CNS have benzodiazepine binding sites, and secondly, if so, are these sites linked to GABA receptors? These questions are addressed in the following section.

2.4.2/ The development of an assay for the binding of [^{3}H]FNZP to the locust ganglionic membrane preparation, P_2G

The centrifugation assay used to measure the binding of [^{3}H]muscimol to P_2G could not be used to investigate the binding of [^{3}H]FNZP in the same tissue (2.3.2.1). Therefore the filtration method used to measure the binding of [^{3}H]FNZP to rat brain membranes (Braestrup & Squires, 1977) was successfully developed using membranes, P_2R, from whole rat brain. Specific, saturable binding of [^{3}H]FNZP to P_2R was observed with a K_D of 6.4 nM (Fig.10) compared with a figure of 3.4 nM reported for [^{3}H]diazepam binding in the original work by Braestrup & Squires (1977). However, no consistent binding of [^{3}H]FNZP was observed in the locust membranes, P_2G, when this filtration assay was used, until the calcium chelating agent, EGTA, was removed from the assay buffer; specific [^{3}H]FNZP binding was then observed in P_2G.

2.4.3/ Effect of Ca^{2+} ions on [^{3}H]FNZP binding in P_2G and P_2R

Specific [^{3}H]FNZP binding to locust ganglionic membranes was abolished in the presence of 1 mM EGTA, and enhanced up to 100% by Ca^{2+} ions, while Mg^{2+} ions had no effect. The concentration of Ca^{2+} ions giving maximal enhancement of [^{3}H]FNZP binding was 1-5 mM. None of these treatments (1 mM EGTA, 4 mM Ca^{2+} or 4 mM Mg^{2+})

had any significant effect on [^{3}H]FNZP binding in rat brain membranes (Figs.11&12). This lack of effect of Ca^{2+} ions on rat brain membrane [^{3}H]FNZP binding is in agreement with other workers who report enhancement of benzodiazepine binding by divalent cations such as nickel (1-5 mM) but report no effect of Ca^{2+} or Mg^{2+} (3.5-100 mM) (Mackerer & Kochman, 1978; Mizuno et al., 1982; Mizuno et al., 1983). However, Lo & Snyder (1983) have reported that the Type II [^{3}H]FNZP binding activity solubilized from bovine brain membranes is enhanced by 5 mM Ca^{2+}, but this effect is masked in the presence of 0.1 M Cl^- which has a similar enhancing effect. Gavish et al. (1985) have reported that 2.5 mM $CaCl_2$ affords 50% protection against heat inactivation of solubilized benzodiazepine receptors from bovine brain, but only in the presence of 500 μM GABA. However, Mizuno et al. (1984) have reported that Ca^{2+} does enhance the binding of [^{3}H]diazepam to solubilized mammalian benzodiazepine receptor but not in membrane fragments, reflecting a possible loss of cation specificity on receptor solubilization. The experiments reported here were performed in 0.16 M Cl^-, which might mask any Ca^{2+} effect on [^{3}H]FNZP binding in rat brain membranes according to Lo & Snyder (1983), although the experiments used membrane fragments rather than solubilized receptor. Above all though, the results presented are from parallel experiments on locust and rat brain membranes and suggest therefore that there is

real difference in the behaviour of the two receptor sites. This could reflect a difference in the specifity of a cation recognition site associated with any GABA/benzodiazepine receptor complex in the synaptic membranes of locust ganglia and rat brain. It would be interesting to investigate the effects of nickel and other divalent cations on [^{3}H]FNZP binding to locust ganglionic membranes.

The difference in effects of cations between rat and locust tissues was further investigated by atomic absorption spectroscopy.

2.4.4/ Measurement of calcium and magnesium levels in locust and rat tissues

Gross calcium and magnesium concentrations of biological tissues can be measured by atomic absorption spectroscopy (eg: the calcium concentration of squid axoplasm, Blaustein & Hodgkin, 1969). Essentially the tissue is ashed to destroy any organic material, the ash is dissolved in acid and the sample is then added to a flame. The radiation absorbed by the atoms can be used as a measure of the concentrations of those atoms in the sample. The sample can either be 'wet' ashed (eg: in a 1:1 mixture of H_2O_2 and H_2SO_4) or 'dry' ashed when a sample is placed in a crucible in a muffle furnace which ensures a controlled temperature and good air flow. Dry ashing is advantageous over wet ashing by being simple, not requiring special apparatus and it is easy to use

for several samples. However dry ashing runs a greater risk of losing elements through their volatilization, though in comparisons of wet and dry ashing the measurement of calcium and magnesium gave good agreement between the two methods (Adrian, 1973; Isaac & Johnson, 1975). Most methods recommend a temperature of 550-700^{o}C for ashing of samples for calcium determination, but 450-500^{o}C for magnesium (Bock, 1976). Because the same sample was to be used for both calcium and magnesium determinations, a compromise of 550^{o}C was used for ashing samples. The crucible in which the sample is ashed is another source of potential error because elements can be adsorbed onto the crucible and not dissolved with acid. Silicates, phosphates and oxides combine readily with the glaze of porcelain crucibles and therefore silica crucibles were used. Though some workers claim that magnesium reacts with silica (Hamilton et al., 1967), others disagree (Adrian, 1973). A further possible error is that unburnt residual carbon can absorb calcium and magnesium but this was avoided by using a long ashing time. Also some elements can be adsorbed onto the glassware used for handling the samples, but this is negligible for calcium and magnesium at pHs below 1.5 (Smith, 1973) and hence concentrated nitric acid was used throughout. Essentially any errors should be minimal and occur in the determination of magnesium rather than calcium, because calcium is less likely than magnesium to be lost

by volatilization at 550°C, or to interact with the crucible.

The level of calcium in rat brain has been reported to be 40 µg/g (McIlwain & Bachelard, 1971) compared with 44.4 µg/g reported here, 50% of which was washed out in the membrane preparation, P_2R. The basal levels of calcium and magnesium which are not removed from the membrane preparations by washing are probably associated with phospholipid; cerebral tissues extracted with organic solvents were shown to have 50% of the total calcium and magnesium associated with diphosphoinositide (Folch et al., 1957). Most measurements of calcium and magnesium in insect tissues have been carried out on whole insects which show high calcium concentrations due to the cuticle and supporting tissues. However, levels have been measured in haemolymph where the calcium concentration varied from 40 µg/ml in the wasp (Vespula) to 280 µg/ml in the silkworm (Bombyx), and the magnesium concentration from 12.8 µg/ml (wasp) to 571 µg/ml (silkworm). Furthermore figures may vary between different ages of the insect as levels of calcium and magnesium decrease during the transition from larva to adult insect (Chapman, 1975). In short the figures presented here (Figs.13&14) are of the same order as reported for haemolymph and the variability in the measurements may be partially accounted for by differences in the ages of the locusts.

With respect to the binding of [^{3}H]FNZP, the

ganglia show a variable concentration of calcium of 130 μg/g which is approximately equivalent to 3 mM. The membrane preparations had variable levels of calcium in the region of 1 mM. Therefore the addition of buffer containing 4 mM $CaCl_2$ would restore the membrane preparations to a calcium concentration in excess of the physiological levels. The washing out of physiological levels of calcium from the rat preparations is easily rectified by the addition of 4 mM $CaCl_2$ and yet no increase in [^{3}H]FNZP binding was observed. As discussed above, locust [^{3}H]FNZP binding exhibits a real difference from the comparable binding in rat brain membranes, with respect to calcium. This calcium effect in locust membranes appears, from the atomic absorption spectroscopy data, to be optimal at physiological calcium concentration.

2.4.5/ The binding of [^{3}H]FNZP to the locust ganglionic membrane fraction, P_2G

Having discovered that specific [^{3}H]FNZP binding could be observed in P_2G by replacement of EGTA with calcium, the characteristics of the binding could be investigated further.

The locust ganglionic membranes show specific saturable binding of [^{3}H]FNZP which is linear with protein concentration over the concentration used in the assay (Fig.15). Diazepam was routinely used as displacing ligand, rather than unlabelled FNZP, due to

availability. However, from the pharmacology experiments (Fig.19) it is clear that the two ligands display very similar profiles with respect to the displacement of [^{3}H]FNZP binding. The standard assay of [^{3}H]FNZP binding was carried out over a 30 min incubation period, after which time over 90% of the specific [^{3}H]FNZP binding has associated (Fig.18).

Housefly thoracic muscle has been reported to have a high affinity (K_D 24 nM) and low affinity (K_D 994 nM) binding site for [^{3}H]FNZP (Abalis et al., 1983), while cockroach bain has a single low affinity site of K_D 383 nM (Lummis & Sattelle, 1986). By Scatchard analysis the specific [^{3}H]FNZP binding in locust P_2G (Fig.16c) exhibits a K_D of 17-47 nM. Derivation of a K_D from the kinetic data gives a value of 26 nM, in good agreement with the equilibrium binding data. According to Chang et al. (1975) the concentration of receptor should be less than 0.1 x K_D for valid Scatchard analysis. If all the binding measured does have a K_D of 17-47 nM then this criterion is satisfied in the experiments reported here. The Scatchard analysis is based on a linear, single site analysis and this is supported by the Hill coefficient (0.91, Fig.16b) which is close to unity. [^{3}H]Diazepam binds to high affinity binding sites in rat brain of K_D 1-4 nM (Braestrup & Squires, 1977; Mohler & Okada, 1977; Braestrup & Squires, 1978), and rat brain also binds [^{3}H]FNZP with high affinity (K_D 0.5 nM, Wastek et al., 1978). The data

presented for locust [^{3}H]FNZP binding cannot be analysed for a binding site of such high affinity owing to the low signal obtained at low [^{3}H]FNZP concentration. However, though the Hill analysis of the equilibrium binding data is indicative of non-heterogeneity, other data on the binding of [^{3}H]FNZP to locust ganglionic membranes do imply some heterogeneity. The secondary analysis of the on-rate data (Fig.18b) exhibits complex kinetics, indicative of heterogeneity of binding sites. Furthermore, when Hill analyses are made of the curves for the displacement of [^{3}H]FNZP by a range of concentrations of unlabelled FNZP, diazepam and Ro5-4864 (Table 5), the Hill coefficients are all below unity, implying that there is heterogeneity (or negative cooperativity) in the binding of [^{3}H]FNZP to P_2G. If then the range of [^{3}H]FNZP concentrations used in the Scatchard analysis of [^{3}H]FNZP binding to P_2G is not wide enough to detect a binding site of higher (or lower) affinity, it would explain why the equilibrium binding data are consistent with a homogeneous population of receptors. However, analysis of such additional binding sites is presently precluded by difficulties in obtaining large quantities of membranes and the relatively low specific radioactivity of the [^{3}H]FNZP.

As pointed out above, derivation of K_D from the kinetic data gives a value of 26 nM. It should be noted though, that this is based on an on-rate derived

from the first five minutes of association (Fig.18b) due to the non-linear form of the secondary plot of the association data and hence this may be an unreliable figure. Also the [^{3}H]FNZP concentration was 15 nM such that even the initial rate of association may have a major contribution from a low affinity site. The apparent heterogeneity in the binding sites implied by the on-rate data can be further analysed by varying the concentration of [^{3}H]FNZP in the association experiments, and the time of preincubation of membranes with [^{3}H]FNZP in the dissociation experiments. In this way one can determine whether the heterogeneity is parallel (due to multiple sites) or sequential (due to different forms of the same site) (Galper et al., 1977). Also use of a lower [^{3}H]FNZP concentration in the on-rate analyses may well allow a better measure of any high affinity binding site. However, this is limited again by the loss of signal at low [^{3}H]FNZP concentrations. Therefore at present it is concluded that locust ganglia contain specific saturable [^{3}H]FNZP binding sites which are probably a heterogeneous population.

2.4.6/ The pharmacology of the binding site for [^{3}H]FNZP in P_2G

The effects of various benzodiazepines on [^{3}H]FNZP binding were investigated in P_2G. For such an analysis, not only should the concentration of receptor be less than 0.1 x K_D (as discussed for Scatchard

analysis), but furthermore the concentration of the labelled ligand should be less than 25% of the concentration of cold ligand which gives 50% displacement of the labelled ligand (Lunt, 1985); ie, for a ligand whose IC_{50} is 100 nM the concentration of labelled ligand should be less than 25 nM. The experiments utilized 5 nM [^{3}H]FNZP which is the lowest concentration which could be used without loss of a significant specific signal.

The pharmacology of the [^{3}H]FNZP binding sites in locust ganglionic membranes (Fig.19) appears to resemble the mammalian peripheral receptor (PBR) as Ro5-4864, FNZP, and diazepam are all at least 100-fold more potent than clonazepam in inhibiting [^{3}H]FNZP binding. Similarly, the [^{3}H]FNZP binding activity of both housefly thoracic membranes (Abalis et al., 1983) and cockroach brain membranes (Lummis & Sattelle, 1986) show pharmacologies which are similar to the mammalian PBRs rather than CBRs. However it should be noted that the differences in potencies in the locusts are small compared with those observed in mammalian preparations (Regan et al, 1981; Mohler et al., 1980). Furthermore Ro5-4864, FNZP and diazepam are equipotent in locust ganglia, whereas Ro5-4864 is 10-fold more potent than FNZP and diazepam in vertebrate peripheral tissues (Regan et al., 1981). Roll-5073 is 100-fold more potent than its enantiomer, Roll-5231, in inhibiting [^{3}H]diazepam binding in rat brain membranes (Mohler et

al., 1980) whereas the two ligands show little difference in their efficacy in insect ganglion, indicating further differences between the insect and mammalian central benzodiazepine sites.

The enhancement of central benzodiazepine binding affinity by GABA receptor agonists has been observed by numerous workers (Tallman et al., 1978; Martin & Candy, 1978; Wastek et al., 1978). Similar behaviour would be expected in the locust ganglionic binding if this binding is linked to GABAergic activity, though mammalian peripheral receptors are unaffected by GABA. GABA has been shown to enhance [^{3}H]FNZP binding in housefly thoracic preparations (Abalis et al., 1983). However this enhancement differed from the comparable enhancement in vertebrate tissues, in that it was dose-dependent, optimal at 70 nM GABA, while no effect was observed with 100 µM GABA. Similarly the [^{3}H]FNZP binding to cockroach brain, observed by Lummis & Sattelle (1986), was enhanced by 49-71% in the presence of 100 nM GABA but only by 15% by 1 µM GABA. Abalis et al. (1983) postulate receptor desensitization as an explanation of their data. GABA and its agonists muscimol and isoguvacine exhibited variable enhancing effects on locust ganglionic [^{3}H]FNZP binding. Enhancement of 15 nM [^{3}H]FNZP binding by GABA and isoguvacine in a dose-dependent manner, optimal at 10 nM (similar to that reported by Abalis et al., 1983) was observed in early experiments but this effect is not

reproducible. Furthermore GABA and muscimol at 10^{-6}-10^{-8} M have been shown to enhance 5 nM [^{3}H]FNZP binding, although the levels of enhancement were not as marked. The most consistent effect is the enhancement of 5 nM [^{3}H]FNZP binding by 10^{-4} M GABA or isoguvacine. This is more reminiscent of the mammalian system where it is the low affinity GABA receptor which mediates GABA enhancement of [^{3}H]FNZP binding (Olsen, 1982). Whether these data represent interchangeable forms of a common receptor or a heterogeneous population of GABA or benzodiazepine receptors which are linked together is unknown. Furthermore, both in the pharmacology experiments and the photoaffinty labelling studies, the effects of GABA on benzodiazepine binding are not always reproducible. There may therefore be an added complication in that the linkage between locust central GABA and benzodiazepine receptors may be quite weak, leading to its loss in some preparations. Another possibility is that if endogenous GABA is not completely removed from the membrane preparation, it may abolish an effect requiring 10^{-8} M GABA, while an effect requiring 10^{-4} M GABA would probably still be observed. What is clear is that some proportion of the benzodiazepine binding observed in locust ganglionic membranes is linked to GABAergic activity, in a manner not directly analogous to the vertebrate central $GABA_A$ receptor.

2.4.7/ Photoaffinity labelling of P_2G with [^{3}H]FNZP

In the case of mammalian benzodiazepine receptors the central receptors differ from their peripheral counterparts in their susceptibility to photoaffinity labelling with FNZP (Thomas & Tallman, 1981; Marangos et al., 1982). In the locust ganglionic preparations two major and two minor proteins are labelled as shown both by fluorography and gel slicing (Figs.23&24) and in this respect the locust benzodiazepine binding activity resembles the mammalian central rather than peripheral type receptors. However, in the same labelling experiments, the locust proteins labelled were of different M_r to the single labelled protein from rat brain (Fig.23).

The variability of benzodiazepine receptor labelling reported by different groups has already been briefly described in the Introduction (2.1.2.1) to this chapter. Essentially the single labelled protein in rat membranes of approximate M_r 50k reported here is in agreement with several other workers (Mohler et al., 1980; Thomas & Tallman, 1981; Carlin & Siekevitz, 1984). The heterogeneous profile of proteins labelled in the locust membranes is different from the most heterogeneous mammalian labelling profile, obtained with hippocampal membranes (Sieghart & Karobath, 1980), where four proteins of M_rs 51k, 53k, 55k and 59k were labelled. Furthermore it was the proteins of M_r 51k and 55k which were most strongly labelled. Indeed only two

proteins of M_r 51k and 55k were reported to be labelled in rat hippocampus in a later paper (Eichinger & Sieghart, 1984).

The only study of [^{3}H]FNZP photoaffinity labelling of brain tissues other than mammalian is the recent work of Hebebrand et al. (1986a). Mouse brain possessed a single labelled protein of M_r 53k, but though an identically sized protein was labelled in calf brain, two other proteins of M_r 56k and 59k were also labelled with equal intensity and a fourth protein of M_r 54k was weakly labelled. Chick brain exhibited two equally labelled proteins of M_rs 53k and 54k (this has been confirmed in other avians, Hebebrand et al., 1986b) and frog brain had a major labelled protein of M_r 53k and a minor labelled protein of M_r 47k. This demonstrates that species variations may exist amongst central benzodiazepine receptors, but furthermore the phylogenetically oldest species tested was a fish (trout) which had two major labelled proteins of M_r 47k and 55k with an intermediate protein of M_r 53k also weakly labelled. In other words the species whose labelling pattern is the most similar to that reported here for locust is, phylogenetically, the oldest species.

Evidently the benzodiazepine binding sites of locust CNS can be photoaffinity labelled with [^{3}H]FNZP, but the labelling pattern is different from any published pattern for photoaffinity labelled

benzodiazepine receptors in mammalian brain. However the locust ganglionic pattern is quite similar to that of fish brain. Whether or not the two major labelled proteins in locust membranes correspond to the two purported benzodiazepine receptors of M_r 51k and 55k, and therefore to Type I and Type II central benzodiazepine receptors as postulated by Hirsch et al. (1985) (see 2.1.2.1), one can not infer at present. This might be further elucidated by repeating the labelling in the presence of Type I receptor-specific ligands such as the β-carbolines, and observing if there is any loss of specifically labelled bands.

An additional explanation of the heterogeneity of benzodiazepine photoaffinity labelling is differing extents of glycosylation of the receptors (Sweetnam & Tallman, 1986). Whether or not this might explain the heterogeneity of labelled proteins in locust membranes is unknown, though one might gain information on this by exposure of the membranes to glycosidases, such as neuraminidase or endoglycosidase-H.

A further possibility is that the four-band labelling pattern obtained with the locust membranes represents proteolytic breakdown products of a single protein. This might be investigated further by the effect of antiproteases on the labelling pattern, though common antiprotease cocktails appear to offer little protection against insect proteases (Lunt, 1986; Cattell, personal communication). If proteolysis has

occurred this would suggest that the M_r of the locust benzodiazepine receptor is at least 57k, which is larger than the protein most commonly labelled from mammalian brain.

The variable effects of GABA on [^{3}H]FNZP binding to P_2G have already been discussed above. The effects of GABA on [^{3}H]FNZP photolabelling are also variable. Though enhancement of 15 nM [^{3}H]FNZP labelling was observed in the presence of 10^{-8} M GABA, no enhancement of 20 nM [^{3}H]FNZP labelling was observed in the presence of 10^{-4} M or 10^{-8} M GABA, and no enhancement of 10 nM [^{3}H]FNZP labelling was observed in the presence of 10^{-4} M GABA. In the binding of [^{3}H]FNZP to locust ganglionic membranes, the effects of 10^{-8} M GABA were not marked and quite variable. Therefore variable enhancement of photoaffinity labelling by 10^{-8} M GABA is not surprising, especially as any residual GABA not washed from the membranes might abolish the effect. However 10^{-4} M GABA had a marked effect on 5 nM [^{3}H]FNZP binding, but no effect on 10 nM [^{3}H]FNZP labelling. The likelihood of an additional high affinity benzodiazepine binding site in the locust ganglionic membranes has already been discussed. If the membranes possess a [^{3}H]FNZP binding site with a K_D in the region of 1 nM (by analogy with mammalian brain, Wastek et al., 1978) that is linked to a low affinity GABA receptor, then at 10 nM [^{3}H]FNZP, this site would be saturated and one would not observe an enhancement of labelling by

10^{-4} M GABA. Therefore future experiments should attempt to photoaffinity label the membranes using 5 nM [^{3}H]FNZP, though the signal would be very low. Sieghart et al. (1983) have pointed out that when investigating allosteric effects on photoaffinity labelling, one should employ short irradiation times because on irradiation, [^{3}H]FNZP becomes irreversibly attached to the receptor and therefore shifts the reversible equilibrium between the ligand and its receptor. Therefore reproducible GABA enhancement of photoaffinity labelling might be observed if shorter irradiation times are employed.

2.4.8/ Stoichiometry of muscimol and benzodiazepine binding sites

The density of high and low affinity [^{3}H]muscimol binding sites in locust brain membranes is 70 & 400 fmol/mg protein respectively. However, the density of [^{3}H]FNZP binding sites in the same tissue preparation is 500 fmol/mg protein and is therefore in excess of the number of high affinity [^{3}H]muscimol binding sites. In the first report of purified bovine brain receptor complex (Sigel et al., 1983) the stoichiometry of [^{3}H]muscimol:[^{3}H]FNZP sites was 3.3-3.8:1, though in an improved preparation with better retention of the regulatory sites of the complex, the ratio was 1.2-2.4:1 (Sigel & Barnard, 1984). Martini et al. (1983) reported a binding ratio of [^{3}H]muscimol:[^{3}H]FNZP of aproximately 1 for a solubilized rat brain preparation. In any case it would appear that there are at least an equal number, if not

more, [^{3}H]muscimol binding sites than [^{3}H]FNZP sites in the mammalian $GABA_A$ receptor complex. This is in contrast to the excess of total [^{3}H]FNZP binding sites compared with [^{3}H]muscimol binding sites in locust brain. Therefore, even if all the observed [^{3}H]muscimol binding is associated with complexes which have associated [^{3}H]FNZP binding activity, one must postulate that there is an additional population of non-GABAergic [^{3}H]FNZP binding sites. We know from considerations of the labelling studies, the association kinetics of binding and Hill analysis of the displacemant of [^{3}H]FNZP binding by cold benzodiazepines that there is a heterogeneous population of benzodiazepine receptors in locust CNS. Furthermore the locust [^{3}H]FNZP binding sites exhibit a mixture of the characteristics of both central and peripheral type benzodiazepine binding sites, as summarized in Table 7. One reservation which should be made when using such a direct mammalian classification of binding sites is that it is not always useful to attempt to classify an insect receptor in terms of criteria derived from studies on mammalian tissue. Indeed in the same locust supraoesophageal ganglion, cholinergic receptors have been described which do not fit precisely into the classical groups of muscarinic or nicotinic (Filbin et al., 1983; Aguilar & Lunt, 1984). However, if one assumes the peripheral and central classification of mammalian benzodiazepine receptors, an explanation of the data presented here is

CHARACTERISTIC	PERIPHERAL-TYPE	CENTRAL-TYPE	LOCUST
TISSUE SOURCE	NEURONAL & OTHER TISSUES EG: KIDNEY	NEURONAL	SUPRAOESOPHAGEAL GANGLION
LIGAND SENSITIVITY	Ro5-4864 - SENSITIVE	CLONAZEPAM - SENSITIVE	Ro5-4864 - SENSITIVE
EFFECT OF GABA	UNAFFECTED BY GABA	BZ BINDING IS ENHANCED BY GABA	BZ BINDING IS ENHANCED BY GABA
PHOTOAFFINITY LABELLING	CAN NOT BE PHOTOLABELLED WITH FLUNITRAZEPAM	CAN BE PHOTOLABELLED WITH FLUNITRAZEPAM	CAN BE PHOTO-LABELLED WITH FLUNITRAZEPAM

Table 7 Comparison of the major characteristics of locust benzodiazepine binding activity with those of mammalian peripheral- and central-type benzodiazepine receptors.

that locust brain possesses benzodiazepine receptors, of which a large proportion are peripheral type and a smaller proportion are central type and are associated with GABA receptors. Two observations support this suggestion. Riley (personal communication) has performed binding assays on the same membrane preparation used for the [^{3}H]FNZP binding assays, but using [^{3}H]Rol5-4513. This ligand is specific for the mammalian central benzodiazepine receptor (Mohler et al., 1984) and shows a level of binding in locust ganglionic membranes which is reduced compared with that of [^{3}H]FNZP, and indeed is comparable with the level of high affinity [^{3}H]muscimol binding. Furthermore one can attempt to estimate the numbers of receptors which are photoaffinity labelled by [^{3}H]FNZP as these should be purely central type (Thomas & Tallman, 1981). One has to assume that, as in the mammalian brain (Mohler et al, 1980), [^{3}H]FNZP only labels 25% of the available sites. A completely accurate estimation cannot be made as the amount of protein loaded on the electrophoretic gel is unknown because tissue is lost during the wash steps prior to loading. However an estimation of the number of sites which are photoaffinity labelled is 5-10 fmol/mg protein, under conditions where one would expect over 100 fmol bound ligand/mg protein in a binding assay. Thus this also suggests that only a proportion of the observed [^{3}H]FNZP binding activity is of central type.

In conclusion, it seems plausible that there

is a population of peripheral type benzodiazepine receptors in locust CNS, in addition to a population (probably aproximately 100-200 fmol/mg protein) of central benzodiazepine receptors which are involved in GABAergic activity.

2.4.9/ A possible GABA receptor complex in insect CNS

In addition to the data presented above on the linkage of benzodiazepine and GABA binding sites in locust CNS, Lummis & Sattelle (1986) have also reported the dose-dependent GABA enhancement of [^{3}H]FNZP binding in cockroach CNS. This GABA/benzodiazepine linkage has been borne out by the work of Beadle and colleagues on current- and voltage-clamped cultured locust neurones; GABA-evoked responses in these cultures are enhanced by 10^{-6} M FNZP (Lees et al., 1985). By analogy with the mammalian $GABA_A$ receptor complex, if insects possess linked GABA and benzodiazepine receptors, do they also possess binding sites for cage-convulsants, such as t-butylbicyclophosphorothionate (TBPS)? If so, are such sites linked to GABA receptors and are they sensitive to picrotoxin or the barbiturates?

Tanaka et al. (1984) have reported the specific binding of picrotoxin to a preparation from cockroch CNS which by Scatchard analysis exhibited a B_{max} of 1.4 pmol/mg protein and a relatively low affinity constant, K_D of 800 nM. More recently Cohen & Casida (1986) have reported binding of the

cage-convulsant, [^{35}S]TBPS to a preparation from whole housefly thorax and abdomen. This binding does not require Cl^- ions for optimal activity, has a B_{max} of 2.5 pmol/mg protein and a K_D of 210 nM, and is only weakly inhibited by picrotoxin (IC_{50}=3.28 x 10^{-5} M). These data are in contrast with the low concentrations of high-affinity [^{35}S]TBPS binding measured in membranes from locust CNS (K_D 10 nM, B_{max} approx. 200 fmol/mg protein, Brown, personal communication), housefly head (K_D 51 nM, B_{max} 220 fmol/mg protein, Szamraj et al., 1986) and cockroach CNS (K_D 18 nM, B_{max} 180 fmol/mg protein, Lummis & Sattelle, 1986). Furthermore the binding in housefly head is greatly reduced in the absence of Cl^- ions. The binding of [^{35}S]TBPS has been observed in torpedo electroplax which is known not to possess any GABA receptors (Abalis et al., 1985a). Thus TBPS is thought to bind to voltage-regulated Cl^- channels in addition to GABA receptor-regulated Cl^- channels (Eldefrawi, unpublished observations). Therefore the possibility arises that [^{35}S]TBPS binding can be contaminated by such a none-GABAergic component and this might be more likely in the study of Cohen & Casida (1986) which uses a very heterogeneous tissue source. Evidently insect CNS possesses binding sites for cage-convulsants, though one observation common to all the binding studies described above is that picrotoxin is not very potent at invertebrate TBPS binding sites.

No enhancement of the binding of [^{3}H]FNZP

reported in this thesis was observed in the presence of 0.1 or 1 mM pentobarbital or picrotoxin under standard conditions (2.3.2.5.5). Supavilai & Karobath (1980) have reported that physiological temperatures are necessary to obtain allosteric interactions of the mammalian $GABA_A$ receptor complex. However, the use of 22°C or 37°C incubations did not result in any effect of pentobarbital or picrotoxin on locust ganglionic [^{3}H]FNZP binding. The cage-convulsant/picrotoxin binding site is the most labile component of the mammalian $GABA_A$ receptor complex (Sigel & Barnard, 1984). It is conceivable therefore, that linkage of any such site in a locust central GABA receptor complex is also highly labile, to the extent that the vigorous membrane preparation abolishes any observable linkage to a benzodiazepine receptor. More recently, however, in this laboratory, the locust central benzodiazepine binding activity has been shown to be enhanced up to 20% by 0.1 mM pentobarbital (Brown, personal communication) and similarly the binding of [^{3}H]GABA in the same tissue is enhanced by 20% by 0.1 mM pentobarbital (Jeffery, personal communication). Both these enhancing effects are not always reproducible, but when present, they are abolished by 1 mM picrotoxin. Also Beadle and colleagues have shown that GABA-evoked responses in current- and voltage-clamped cultured locust neurones are enhanced by 10^{-4}-10^{-5} M pentobarbital (Beadle et al., 1985). However, these GABA responses are also completely

blocked by 10^{-6} M picrotoxin (Lees et al., 1985). These effects of picrotoxin are in contrast with the weak efficacy of this ligand described above in [^{35}S]TBPS binding assays, possibly reflecting a close association of a picrotoxin site with the barbiturate site rather than the cage-convulsant site of any insect central GABA receptor complex.

The [^{35}S]TBPS binding observed by Cohen & Casida (1986) is enhanced by 40% by 10-100 μM GABA, in contrast with that observed by Lummis and Sattelle (1986) which is inhibited by 1 μM GABA.

In conclusion, the last four years have seen a burst of interest in insect central GABA receptors from which we are just beginning to reap the rewards. Evidence is accumulating that insect CNS possesses binding sites for GABA, benzodiazepines and TBPS and that a proportion of these different binding activities are linked to each other, probably in a complex analogous in overall organization with the mammalian $GABA_A$ receptor. The detailed pharmacology of such insect central GABA complexes is still to be elucidated, but the present implications are that they differ significantly from their mammalian central counterparts.

2.4.10/ On the evolution of GABA and benzodiazepine receptors

Quite recently it was thought that central benzodiazepine receptors (Nielsen et al., 1978) and bicuculline-sensitive GABA receptors (Mann & Enna, 1980) evolved with the vertebrates. However, Nielsen and colleagues did not use Ca^{2+} in their benzodiazepine binding assays, which might explain the lack of benzodiazepine binding in invertebrate brain. Also it is now well established, as discussed above, that insect central GABA receptors are relatively insensitive to bicuculline. Therefore, contrary to the conclusions of Simmonds (1983) that invertebrates possess 'simple' GABA receptors which lack any associated allosteric sites, evidence is now accumulating that a GABA receptor complex, comparable with the $GABA_A$ receptor complex of mammalian brain, is present in the brain of the phylogenetically older insects. We can only speculate whether such receptor complexes appeared in insect brain at the same time as the vertebrates evolved, or whether the evolutionary ancestors of today's insects also possessed GABA receptor complexes. Hebebrand et al. (1986a) reported that phylogenetically older species, as far back as the fishes, have fewer central benzodiazepine receptors, compared with mammalian brain. From the discussion above it is evident that any insect central GABA receptor complexes are present in greatly reduced numbers compared with their counterparts in

vertebrate brain. One must therefore postulate some evolutionary pressure leading to an increased density of central complex GABA receptors which occurred with the arrival of the vertebrates. Whether or not this reflects the evolution of higher brain functions is unknown.

A further consideration to arise from the work in this chapter is that of the evolution of the peripheral benzodiazepine receptors. Bolger et al. (1985) have postulated a very late evolutionary appearance of peripheral type benzodiazepine receptors, implying highly specialized functions for these receptors in the central nervous system. However, we now know that insect CNS possesses significant concentrations of Ro5-4864-sensitive [^{3}H]FNZP binding sites (this thesis; Lummis & Sattelle, 1986), implying that on the contrary, peripheral benzodiazepine receptors have an old ancestry. The complex question of the function of peripheral type benzodiazepine receptors in mammalian brain has already been briefly addressed (2.1.2.2). If these receptors do have an evolutionarily old history, perhaps their central function will prove to be a very basic one.

2.4.11/ Future research into insect central GABA receptors

Work should continue to elucidate the detailed pharmacology and linkage of the different GABAergic binding activities which have already been described. However, the use of locust ganglionic membrane preparations is not economical, particularly when using [^{3}H]muscimol whose low specific radioactivity dictates the use of high protein concentrations. Indeed any improvements in the specific radioactivity of radiolabelled GABAergic ligands would be welcome. Perhaps a more economical tool for elucidating the details of insect GABAergic binding site linkage is the electrophysiological recording from cultured insect central neurones (Beadle, 1986).

Photoaffinity labelling studies with [^{3}H]FNZP have already given indications of the size of the peptides involved in insect GABA receptors. Photoaffinity labelling of locust ganglionic membranes with [^{3}H]muscimol (Asano et al., 1983; Cavalla & Neff, 1985) in parallel with [^{3}H]FNZP should demonstrate any commonly labelled peptides. However, again the low specific activity of [^{3}H]muscimol, combined with the low density of binding sites may well preclude such an experiment at present. A problem with the use of [^{3}H]FNZP in photoaffinity labelling studies is that it only labels 25% of the available sites (Mohler et al., 1980). Therefore [^{3}H]Ro15-4513, which labels 100% of the

available sites (Mohler et al., 1984) may be a better ligand to use, though at present the radiolabelled ligand available does not have a very high specific radioactivity. If one could do labelling studies on locust membranes at lower labelled ligand concentrations this might permit a more clear-cut demonstration of GABA linkage, and analysis of the effects of the β-carbolines on the labelling profile.

A further problem in using [^{3}H]FNZP in the binding studies with locust membranes is that it binds to both central and peripheral type binding sites. Havoundjian et al. (1986) have recently demonstrated that mammalian peripheral and central benzodiazepine receptors behave differently in response to treatment with phospholipase A_2, and such treatment may generate information on the different populations of benzodiazepine receptors in insect brain.

All these strategies involve receptors which are still in their membrane environment. A natural progression, by analogy with the mammalian $GABA_A$ receptor complex, is to attempt to solubilize the receptors from locust ganglionic membranes and subsequently purify them. However, the low densities of receptors would dictate the use of considerable quantities of tissue and therefore any such purification experiments are cost-prohibited.

Two further strategies for the study of insect GABA receptors are the use of monoclonal antibodies

(mAbs) and molecular biology. However, to commence with either technique using insect tissue would require purified receptor, either to obtain sequence data to synthesize probes with which to screen for homology in a locust cDNA library, or to immunize mice to raise monoclonal antibodies to the receptors. An alternative is to use the mAbs and molecular biological probes being developed in the mammalian GABAergic field. Use of the mAbs raised against mammalian $GABA_A$ receptor complex might show specific cross-reactivity in sections of locust ganglia, in which case they might be used in immunoblotting experiments with locust ganglionic membranes. Furthermore any antibodies showing cross-reactivity with locust material might be used to screen the expression products of a locust ganglionic cDNA library (though polyclonal antibodies might be of more use than mAbs as they recognise a wider range of epitopes). Similarly, when DNA sequences are available which code for the gene sequence of the mammalian $GABA_A$ receptor complex, these could be used as probes to screen for homology in locust ganglionic cDNA libraries. In this way we might begin to obtain information on the homology between insect and mammalian GABA receptors, and even of the protein sequence of the insect receptors. Otherwise, without considerable help and cooperation from workers in the field of mammalian GABA receptors, it will be difficult to extend our knowledge of insect GABA receptors beyond the level of interacting binding sites in a membrane environment.

CHAPTER 3

STUDIES ON LOCUST SYNAPTOSOMES

3.1/ INTRODUCTION

Following the interaction of GABA with its post-synaptic receptor, the neurotransmitter is cleared from the synaptic cleft by uptake into neurones and glial cells (Iversen & Kelly, 1975). Despite the first demonstration of this being made in an invertebrate preparation (Iversen & Kravitz, 1966), the subsequent characterization of GABA uptake was carried out using mammalian nervous tissue, largely due to the development of experimental procedures that use synaptosomes.

As a result of studies on the subcellular distribution of acetylcholine and its synthetic enzyme, choline acetyltransferase, following discontinuous sucrose density gradient centrifugation of brain homogenates, two groups independently reported the production of pinched-off nerve terminals or synaptosomes from mammalian brain (Gray & Whittaker, 1962; De Robertis et al., 1962). Synaptosomes have since been produced by numerous other techniques and have seen extensive use in neurochemical research (Iversen, 1971; Kuhar, 1973; Whittaker, 1973; Bennett et al., 1974; Jones, 1975; Gibson & Blass, 1982; Whittaker, 1984).

3.1.1/ GABA uptake and release in mammalian brain

Initially brain homogenates and slices were used in the study of GABA uptake (Iversen & Neal, 1968;

Iversen & Johnstone, 1971; Bond, 1973; Lajtha & Sershen, 1975), but the advent of synaptosomal preparations from mammalian brain permitted the study of GABA uptake in the isolated nerve ending (Kuriyama et al., 1969; Martin & Smith, 1972; Levi & Rateiri, 1973; Snodgrass et al., 1973; Sellstrom et al., 1976). These studies were in general agreement that GABA uptake occurred via an active transport system which was saturable, both temperature- and sodium-dependent and inhibited by ouabain (which inhibits Na^+K^+-ATPase). Kanner (1978) took membrane vesicles derived from osmotically shocked synaptosomes and demonstrated that an artificial electrochemical gradient ($[Na]_o > [Na]_i$) plus a low concentration of chloride ion were sufficient to drive the GABA uptake process. The author concluded that though Na^+K^+-ATPase is responsible for maintaining the appropriate electrochemical gradient in the synaptosome, it is the gradient that actually drives the GABA uptake process.

It is generally accepted that GABA uptake in mammalian brain preparations occurs via two systems of high and low affinity (see Levi & Rateiri, 1973). However Wood & Sidhu (1986) have recently postulated that in fact there are three uptake systems of low, medium and high affinity, although the high and medium affinity systems may represent one transport protein molecule, or complex, with two sites for GABA and exhibiting negative cooperativity.

Once synaptosomes could be preloaded with radiolabelled GABA, it was demonstrated that GABA could be released by electrical pulses or K^+-stimulation in a largely Ca^{++}-dependent manner (Bradford, 1970; De Belleroche & Bradford, 1972; Osborne et al, 1973). The major remaining controversy is over whether this GABA release is by a cytosolic release mechanism (De Belleroche & Bradford, 1977; Levi et al, 1978; Abe & Matsuda, 1983) or from a non-cytosolic compartment (Levy et al., 1973; Cotman et al., 1976; Haycock et al., 1978).

3.1.2/ The development of insect synaptosomes and insect GABA uptake

Membrane fractions from the muscle of an arthropod, the crayfish, have been shown to exhibit sodium-dependent, high-affinity GABA uptake (Meiners et al., 1979). However, owing to a lack of any insect synaptosomal preparations, investigations of GABA uptake in insect CNS were limited for a long time to autoradiographic studies (Frontali & Pierantoni, 1973; Hue et al., 1982) as discussed in Chapter 1.

In 1970, Telford & Matsumura reported that the techniques used to produce synaptosomes from mammalian brain (whereby fractionated tissue is applied to a discontinuous sucrose gradient) were of very limited use when applied to cockroach nervous tissue. Because insect synaptosomes might be osmotically sensitive, Donnellan

et al. (1976), working with fleshfly heads, used discontinuous Ficoll gradients rather than sucrose in an attempt to keep the separation conditions isotonic with the synaptosomes. This resulted in a synaptosomal fraction but this fraction was highly contamimated with mitochondria. Dowdall & Whittaker (1973) had already noted that squid head ganglion synaptosomes were buoyant in Ficoll gradients. This concept of flotation led Breer & Jeserich (1980) to publish a microscale flotation technique for the production of synaptosomes from Locusta migratoria. This preparation is based on the flotation of the synaptosomes from sedimenting mitochondria in a continuous Ficoll gradient. The authors stress that the success of their technique is dependant on using a mild hand homogenization, strictly isotonic conditions and a continuously cooled microhomogenizer. The preparation has subsequently been characterized with enzyme markers, ultrastructural analyses and lipid analysis (Breer, 1981a, 1982a).

Synaptosomes produced from insect CNS by the method of Breer & Jeserich (1980) have seen limited use, largely in the study of cholinergic neurotransmission. Synaptosomes derived from locust CNS exhibited high and low affinity [^{3}H]choline uptake; the high affinity [^{3}H]choline uptake was inhibited by hemicholinium-3 and absolutely dependent on external sodium (Breer, 1982b). Synaptosomes loaded with [^{3}H]choline in this way released [^{3}H]acetylcholine on exposure to elevated K^+

concentrations (Breer & Knipper, 1984). Using a similar method to that of Breer & Jeserich (1980), Dwivedy (1985) has reported the production of synaptosomes from the CNS of the cockroach, Periplaneta americana, which also exhibit high affinity, sodium-dependent, hemicholinium-3-sensitive uptake of [^{3}H]choline.

Insect CNS is known to possess a high content of acetylcholine and acetylcholine receptors (Breer, 1981b) and therefore it is not surprising that the availability of insect synaptosomes rapidly led to the elucidation of cholinergic uptake and release mechanisms in insect CNS. However, insect synaptosomes have seen more limited use in the study of GABAergic neurotransmission. Gordon et al. (1982) used the method of Breer & Jeserich (1980) to obtain a synaptosomal fraction from locust nervous tissue, and demonstrated that these synaptosomes could accumulate [^{3}H]GABA. The further study of this uptake utilized synaptosomes which had been osmotically shocked to produce membrane vesicles. The [^{3}H]GABA uptake of these vesicles was Na^+- and Cl^--dependent, abolished by nigericin which would collapse a Na^+ gradient (out > in) and stimulated by valinomycin which should enhance the membrane potential (interior negative). Therefore it appeared that the [^{3}H]GABA uptake capacity of this locust preparation exhibited a similar electrochemical dependence to the corresponding uptake system of mammalian brain. More recently locust synaptosomes have been shown to possess

high and low affinity uptake systems for [^{3}H]GABA (Breer & Heilgenberg, 1985) and synaptosomes from cockroach CNS have also been shown to accumulate [^{3}H]GABA (Whitton et al., 1986).

There is therefore limited evidence that synaptosomes can be produced from insect CNS and that they can be used in the study of GABA uptake. A synaptosomal preparation from locust supraoesophageal ganglion was therefore developed and the physical parameters required for the optimal physical stability of the synaptosomes were characterized by [^{3}H]choline uptake. This preparation was then used in a preliminary study of [^{3}H]GABA uptake.

3.2/ MATERIALS & METHODS

MATERIALS

All reagents and standard enzymes were obtained from Sigma Chemical Corporation or BDH Chemicals Ltd unless otherwise stated. Two-week old adult locusts (Schistocerca gregaria), in their 5th instar, were used in all experiments. Locusts were supplied by the Welsh Moutain Zoo, Colwyn Bay, Wales. [^{3}H]Choline (78 Ci/mmol) and [^{3}H]GABA (54 Ci/mmol) were obtained from Amersham International plc, England. Soluene Tissue Solubilizer was supplied by The Packard Instrument Co., and filters by either Whatman Ltd, England or Millipore, England.

All measurements of radioactivity were made in either an LKB 1217 Rackbeta liquid scintillation counter or a Packard Minaxi Tri-Carb 4000 Series liquid scintillation counter. All radioactivity was measured over a 2 min counting period in OptiPhase 'Safe' scintillant (LKB Scintillation Products, Fisons plc, England). The counting efficiency was 35% and quenching was corrected for.

METHODS

3.2.1/ Assay of lactate dehydrogenase

Lactate dehydrogenase (LDH, EC 1.1.1.27) was assayed by the method of Johnson (1960), as modified by Marchbanks (1967). Occluded LDH activity was measured by the addition of TritonX-100 to a final concentration of 0.3% (v/v). Membrane-bound activity was measured by the addition of 0.5 M NaCl to the assay buffer.

3.2.2/ Assay of glyceraldehyde 3-phosphate dehydrogenase

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) was assayed by the method of Cori et al. (1948). Occluded GAPDH activity was measured by the addition of TritonX-100 to a final concentration of 0.1% (v/v).

3.2.3/ Assay of malate dehydrogenase

Malate dehydrogenase (MDH, EC 1.1.1.37) was assayed spectrophotometrically, following the oxidation of NADH at 340nm (E_{340} 6,200 $lmol^{-1}$ cm^{-1}). Occluded MDH activity was measured by the addition of TritonX-100 to a final concentration of 0.1% (v/v).

3.2.4/ Preparation of purified synaptosomes from locust supraoesophageal ganglia

Initial attempts to isolate synaptosomes from locust followed the method of Breer & Jeserich (1980) (Fig.27). The heads were removed from 50 insects and stored on ice. The cuticle of the head was shaved off and the supraoesophageal ganglion removed onto pre-chilled aluminium foil. Fifty ganglia were pooled and weighed for an initial study. The heads were homogenized (10% w/v) in 0.1 M Tris-HCl, pH 7.3, containing 0.25 M sucrose, using 10 passes of a chilled Jencons glass mini-homogenizer at 4°C. The homogenate was centrifuged at 1200 x g for 15 min at 4°C, and the resulting supernatant, S_1, stored on ice. The pellet was washed twice (buffer and centrifugation as above) and the two resulting supernatants pooled, added to S_1, and the total supernatants were then centrifuged at 15,000 x g for 30 min at 4°C. The resulting pellet, P_2, was resuspended (1/5, v/v) in 0.1 M Tris-HCl, pH 7.3, containing 0.25 M sucrose and 12% (w/v) Ficoll. The mixture was centrifuged at 10,000 x g for 40 min at 4°C in a Sorvall RC-5B superspeed centrifuge. This should result in a mitochondrial pellet and a synaptosomal pellicle at the surface which is removed with a micropipette.

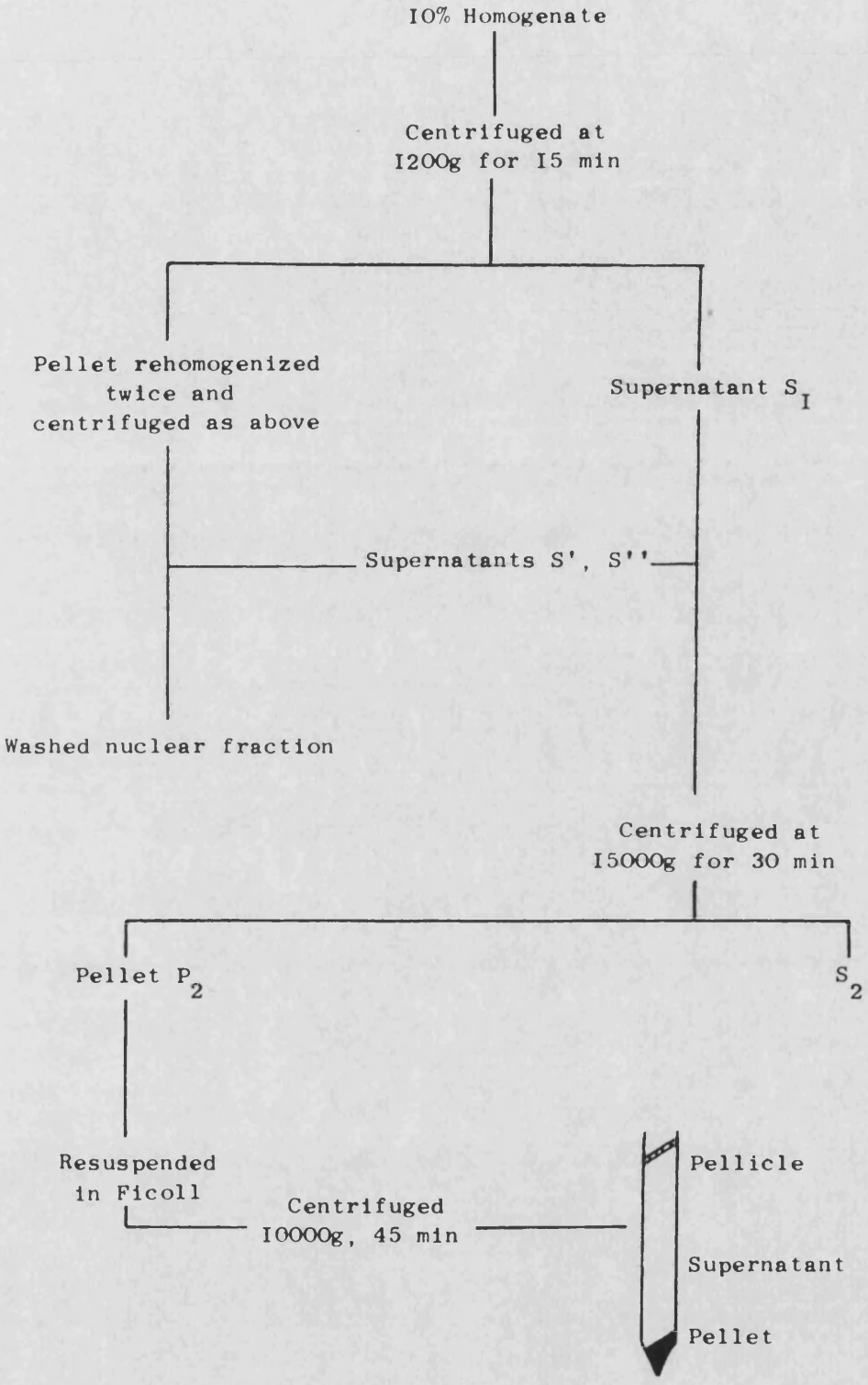


Fig.27 Preparation of purified insect synaptosomes after the method of Breer & Jeserich (1980).

3.2.5/ Preparation of a synaptosome-rich homogenate, H, from locust supraoesophageal ganglia

The supraoesophageal ganglia were removed from 15 adult locusts into 550 µl of chilled buffer PCS (100 mM sucrose, pH 7.4, containing 150 mM NaCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 4 mM $NaHCO_3$ and 6 mM KH_2PO_4). This suspension was homogenized on ice in a 1 ml glass mini-homogenizer (Jencons Scientific Ltd., Bedfordshire, England) using 5 very gentle strokes of a teflon pestle (4.82 mm diameter). This homogenate was either used directly, H, or filtered through nylon bolting cloth (192 µm mesh) and the cloth washed with a further 250 µl of cold buffer PCS (HF). The homogenate typically had a protein concentration of 2 mg/ml.

3.2.6/ Preparation of rat brain (striatal) synaptosomes

Two male Wistar rats (200-250 g) were killed by cervical dislocation and the striata dissected out into chilled 0.32 M sucrose. The striata were then homogenized in a pre-cooled Potter-Elvejham teflon/glass homogenizer (2 x 6 passes). The homogenate was loaded onto two discontinuos sucrose gradients, comprising 0.32 M, 0.8 M, and 1.2 M sucrose. The gradients were centrifuged at 100,000 x g for 1 h at 4°C in a Beckman SW50.1 rotor. The synaptosomes form a visible band at the 0.8 M/1.2 M sucrose interface and were removed and centrifuged at 2000 x g in an MSE bench centrifuge. The pellet was resuspended in 0.32 M sucrose for electron

microscopy.

3.2.7/ Preparation of fractions for electron microscopy

The morphologies of the locust supraoesophageal ganglion and the synaptosome-rich homogenate (3.2.5) were examined by electron microscopy. Table 8 outlines the procedure used to prepare and visualise the tissue.

3.2.8/ Negative staining of samples for electron microscopy

Samples to be stained were in suspension form. A drop of suspension was placed onto a copper, pioloform-coated grid and the grid then removed from the suspension. Excess fluid was drawn off the grid with a filter paper, and the grid dried under a lamp for 10 min. A drop of ammonium molybdate solution was placed on the grid, and the grid removed and dried as previously. The sample was then visualised under the electron microscope as in 3.2.7. The ammonium molybdate solution must be iso-osmotic with the sucrose concentration of the sample suspension to avoid disruption of the synaptosomes; 2.5% (w/v) ammonium molybdate, pH 7.4 for mammalian samples, 3.5% (w/v), pH 7.3 for invertebrate samples. These values are obtained from the calibration curve adapted from Muscatello & Horne (1968) (Fig.28).

Table 8 Procedure for the preparation of samples for electron microscopy

* Samples can be held for longer (2 days) at these stages.

STAGE	CONDITIONS	TIME
Prefix	2.5% Glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (buffer), 4°C	Overnight
Wash	Buffer*	3 x 10 min
Postfix	1% Osmium tetroxide in buffer, room temp	60 min
Wash	Buffer	3 x 10 min
Staining	1% aqueous uranyl acetate in dark	60 min
Wash	Buffer	3 x 10 min
Dehydration	30%, 50%, 70%*, 95% Acetone 100% Acetone	2 > 5 min 3 x 20 min
Embedding	Taab E M Resin 50% resin/50% acetone 100% resin	Overnight 2 x Overnight
Polymerization	60°Coven in Beam capsules	2 - 3 days
Sectioning	Ultrathin sections, LKB microtome on U3	
Staining	Uranyl acetate lead citrate	5 min 10 min
Wash	Buffer	3 x 10 min
Observations	Jeal 100 x C microscope	

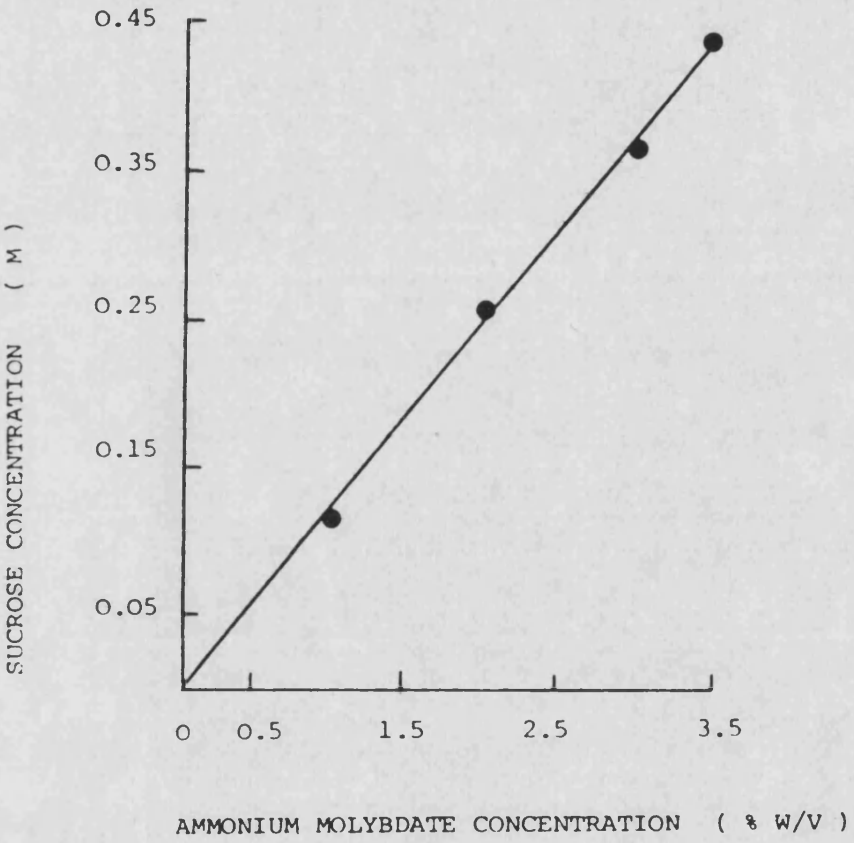


Fig.28 Calibration curve for negative staining of biological samples in sucrose solution with iso-osmotic ammonium molybdate solution, from Muscatello & Horne (1968).

3.2.9/ Assay of [^{3}H]choline uptake by H and HF

To measure the time course of [^{3}H]choline uptake, 90 µl of H or HF (3.2.5) were added to 900 µl of chilled buffer PCS containing 11.1 mM glucose, and then preincubated at 30°C for 7 min prior to the addition of 10 µl [^{3}H]choline (1 mCi/ml, 7 µM final concentration of choline). At set time points 100 µl samples were removed into 1 ml ice-cold buffer PCS and filtered very gently through cellulose nitrate filters (0.45 µm pore size). The filters were then washed with 9 ml ice-cold buffer PCS, dried at room temperature, solubilized with 1 ml 2-ethoxy ethanol and the radioactivity counted following the addition of 5 ml scintillant.

In some experiments individual uptake incubations were used which were exactly as described above but using proportionally reduced volumes. Therefore 9 µl H were added to 90 µl buffer PCS containing 11.1 mM glucose and 1 µl [^{3}H]choline added following preincubation. The mixture was then incubated for 30 min at 30°C and the whole volume then filtered and the radioactivity on the filter counted as described above.

3.2.10/ Release of radioactivity from HF preloaded with [^{3}H]choline

Samples (54 µl) of HF (3.2.5) were preloaded with [^{3}H]choline as described for individual uptake incubations (3.2.9). However following incubation at

30°C for 30 min, rather than filtering, the uptake was terminated by dilution with 13 ml ice-cold buffer PCS and centrifugation for 5 min at 2500 rpm in an MSE bench centrifuge. The resulting pellet was resuspended in 1 ml of either Na^+-buffer (buffer PCS) or K^+-buffer (buffer PCS containing 100 mM NaCl and 50 mM KCl rather than 150 mM NaCl), incubated at 30°C for 5 min and then centrifuged as above. The pellets were solubilized with 200 µl Soluene Tissue Solubilizer and then neutralized with 200 µl glacial acetic acid. The radioactivity of both the supernatants and the solubilized pellets was counted to give a measure of the K^+-evoked release of radioactivity.

In some experiments HF was preloaded with [^{3}H]choline using four-fold greater volumes throughout. Following the first centrifugation the pellets were resuspended in 2.2 ml of either Na^+-buffer or K^+-buffer, both containing the acetylcholine esterase inhibitor, neostygmine (50 µM). Following the second centrifugation a 0.2 ml sample of the supernatant was removed to measure the radioactivity and choline and acetylcholine were extracted from the remainder and analysed by thin layer chromatography.

3.2.11/ Extraction of choline and acetylcholine from aqueous samples

Following the release of radioactivity from [^{3}H]choline-loaded samples of HF (3.2.10) choline (Ch) and acetylcholine (ACh) were extracted from the resulting supernatants by the method of Fonnum (1969). Supernatants (2 ml) were rapidly mixed, sequentially, with 400 µl 3 N perchloric acid, 400 µl 1 M Na_2HPO_4 and 4 ml 2-heptanone, containing 15 mg/ml tetraphenylboron. The mixture was then shaken for 20 sec to extract the choline species into the organic phase, and the organic and aqueous phases were then separated by centrifugation at 2000 rpm in an MSE bench centrifuge. The aqueous phase underwent a second extraction with a further 1 ml heptanone containing 45 mg/ml tetraphenylboron. The choline species were then extracted from the combined heptanone with 5 ml 1 M HCl and this aqueous phase was then washed with 2 x 5 ml heptanone to remove any residual tetraphenylboron. The resulting aqueous phase was freeze-dried overnight in an Edwards Modulyo freeze drier. Typically these lyophilized samples contained a brown residue owing to residual sucrose from buffer PCS and it was therefore necessary to re-extract the choline species prior to thin layer chromatography. The samples were resuspended in 100 µl H_2O and then re-extracted with 600 µl heptanone containing 10 mg/ml tetraphenylboron followed by 600µl 1 M HCl which was then washed twice with heptanone and freeze dried prior to analysis by

thin layer chromatography.

3.2.12/ Thin layer chromatography of choline species

The separation of choline and ACh by tlc employed the method of Hemsworth & Morris (1964) as described by Marchbanks & Israel (1971). Lyophilized samples were reconstituted in 15 µl methanol containing 5 mM Ch and 5 mM ACh as carriers and then spotted onto a 0.1 mm thick cellulose plate (20 x 20 cm, Merck, Darmstadt, W.Germany) which had been pre-run in an 8:2 butanol:ethanol mixture and activated for 10 min at 60°C. The loaded plate was then run in a butanol:ethanol:acetic acid:water (8:2:1:3) solvent system until the front approached the top of the plate. Following drying, the plate was developed by spraying with potassium iodoplatinate; Ch develops a blue colour and ACh a purple colour. The developed spots were scraped into scintillation vials and decolourized with 100 µl 5% (w/v) Na_2SO_3 in ethanol plus 250 µl 0.1 M ethanolic ammonia. The radioactivity was then counted in 10 ml scintillant.

3.2.13/ Assay of [^{3}H]GABA uptake by HF

To measure the time course of [^{3}H]GABA uptake by HF (3.2.5), 225 µl HF were added to 2.225 ml buffer PCS, containing 11.1 mM glucose and then preincubated at 30°C for 7 min. A solution of [^{3}H]GABA and the GABA-T inhibitor, aminooxyacetic acid (AOAA) (50 µl) was then

added to give a final concentration of 50 µM AOAA and 0.1 µM [^{3}H]GABA. Samples (200 µl) were then removed at set time points into 1 ml ice-cold buffer PCS and very gently filtered through 0.45 µm pore size cellulose nitrate filters (Whatman) which were washed, solubilized and the radioactivity measured as described above for [^{3}H]choline uptake (3.2.9). The effects of 0.1 mM nipecotic acid and 0.1 mM 2,4-diaminobutyric acid were studied by incorporating them in the 2.225 ml buffer PCS plus glucose.

3.2.14/ Release of radioactivity from HF preloaded with [^{3}H]GABA

a) Centrifugation method

Individual incubations (90 µl HF in 900 µl buffer PCS plus 11.1 mM glucose) were preincubated in bench centrifuge tubes at 30^{o}C for 7 min before the addition of 20 µl [^{3}H]GABA (0.1 µM final concentration), and then incubated at 30^{o}C for 10 min. The samples were then centrifuged and treated as described above for the release of radioactivity from [^{3}H]choline loaded synaptosomes (3.2.10) except that the washed [^{3}H]GABA loaded samples were resuspended in Na^+-buffer, K^+-buffer or K^+Ca^{2+}-buffer (Table 9).

b) Filtration method

After loading HF with [^{3}H]GABA as described above in 3.2.14a, rather than centrifuging the samples, they were applied to 0.45 μm pore size cellulose nitrate filters (Whatman). The filters were washed twice with 5 ml buffer PCS, before being treated with 2 ml of Na^+-buffer, K^+-buffer or K^+Ca^{2+}-buffer (Table 9) and the filtrate collected. The filters were solubilized and the radioactivity counted as described above (3.2.9). The radioactivity of the filtrates was counted in 18 ml scintillant to give a measure of the radioactivity released by different stimuli.

Buffer	Concentration (mM)		
	NaCl	KCl	$CaCl_2$
Na^+ (PCS)	150	0	4
K^+	106	50	0
K^+Ca^{2+}	100	50	4

Table 9 Summary of differences in ionic content of Na^+-, K^+-, and K^+Ca^{2+}-buffers used in [^{3}H]GABA release studies.

3.3/ RESULTS

3.3.1/ Enzyme activities in locust ganglia

Any successful attempts to produce synaptosomes should result in membrane-enclosed bodies which contain the cytosolic constituents of the nerve terminal. Therefore assay of cytosolic enzyme activities in the synaptosomal preparation, under conditions where both free and occluded enzymes can be measured, may confirm the presence of synaptosomes.

Locust supraoesophageal ganglia were extensively homogenized and the activities in the resulting homogenate of the three enzymes malate dehydrogenase (MDH), lactate dehydrogenase (LDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were assayed (3.2.1-3) in the presence and absence of TritonX-100. The results are compared in Table 10.

GAPDH is assayed in Tris-HCl buffer, pH 7.6 containing 20 mM sodium arsenate whereas the synaptosome preparation buffer is $KH_2PO_4/NaHCO_3$, pH 7.4, containing 100 mM sucrose. Therefore the activity of GAPDH was further analysed with respect to the assay conditions and in particular the assay buffer used, the pH of the assay buffer and the presence and absence of sucrose and arsenate. The results are presented in Table 11.

ENZYME	ACTIVITY (µmol/min/mg protein)		% NON-OCCLUDED
	- TRITON	+ TRITON	
MDH	2.045 ± 0.103	2.714 ± 0.076	75.3 ± 2.5
LDH	0.019 ± 0.001	0.019 ± 0.001	100.0 ± 0.0
GAPDH	0.068 ± 0.007	0.068 ± 0.007	100.0 ± 0.0

Table 10 The activities of the enzymes malate dehydrogenase (MDH), lactate dehydrogenase (LDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in homogenates of locust ganglia in the presence and absence of TritonX-100. Results are from four separate homogenates ±SEM, using duplicate determinations in each case.

BUFFER	SUCROSE	pH	AsO_4	% STANDARD GAPDH ACTIVITY
TRIS-HCL	-	7.6	+	100.0
PHOSPHATE/ CARBONATE	+	7.4	+	62.5
TRIS-HCL	+	7.6	+	95.8
TRIS-HCL	+	7.4	+	77.5
TRIS-HCL	+	7.2	+	68.8
TRIS-HCL	+	7.4	-	1.7

Table 11 Effects of various changes in buffer conditions on standard GAPDH activity in homogenates of locust ganglia. Results are the means of determinations in three separate homogenates. Standard GAPDH activity (100%) is the activity in 80 mM Tris-HCl buffer, pH 7.6, in the presence of 20 mM arsenate and absence of 100 mM sucrose.

3.3.2/ Preparation of synaptosomes from locust supraoesophageal ganglia

Initial attempts to produce synaptosomes from locust supraoesophageal ganglia followed the method of Breer & Jeserich (1980) (3.2.4). The activity of LDH (total and occluded) was measured in various fractions of the purification, and the results are presented in Table 12.

Following the lack of success in producing synaptosomes by this methodology, it was decided to develop, systematically, a procedure for the production of locust ganglionic synaptosomes and to monitor each stage with a morphological assay.

3.3.2.1/ NEGATIVE STAINING OF SYNAPTOSOMES

A rapid, "same-day" electron microscopic monitor for synaptosomes was developed by using negative staining (3.2.8). The procedure was developed using an established preparation of synaptosomes from rat striatum (3.2.6), and typical profiles of these synaptosomes obtained on negative staining are shown in Fig.29.

Locust ganglia were homogenized as described (3.2.5) and on negative staining this preparation exhibited synaptosomal profiles as shown in Fig.30.

Table 12 The activity of LDH in various fractions of the purified synaptosome procedure of Breer & Jeserich (1980). The fractions correspond to those underlined on the inset fractionation procedure. Occluded activity was derived from the activity released by the addition of TritonX-100. Results are the means of four separate experiments ±SEM.

FRACTION	% RECOVERY OF LDH ACTIVITY	% OCCLUDED LDH ACTIVITY
HOMOGENATE	100	47.5 ± 5.3
P_2	37.3 ± 3.0	16.5 ± 2.6
PELLICLE	4.9 ± 2.2	8.0 ± 4.5
SUPERNATANT	15.0 ± 5.4	50.0 ± 28.9
PELLET	2.4 ± 1.5	30.3 ± 15.5

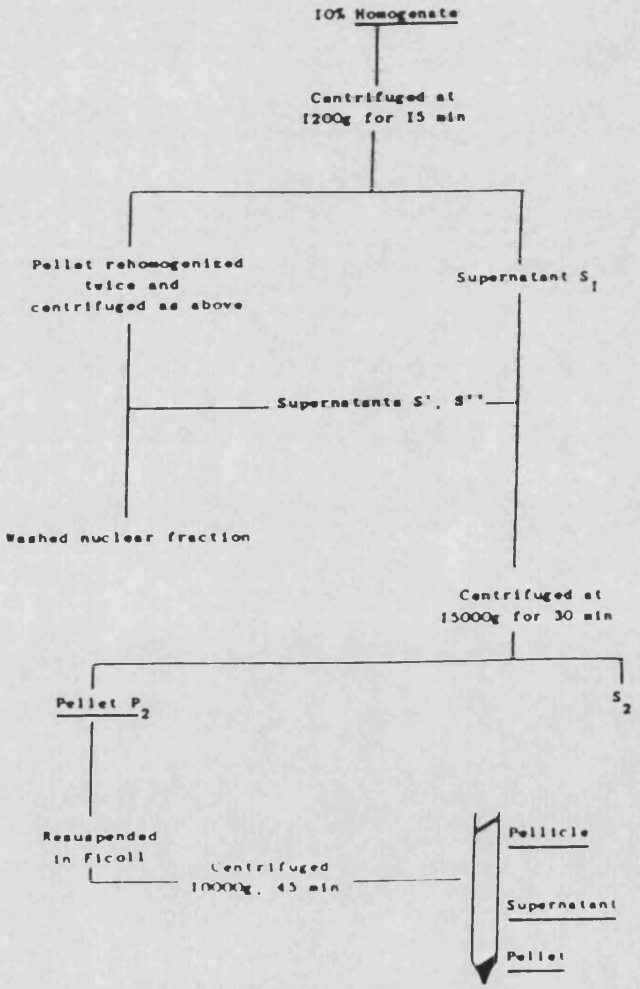
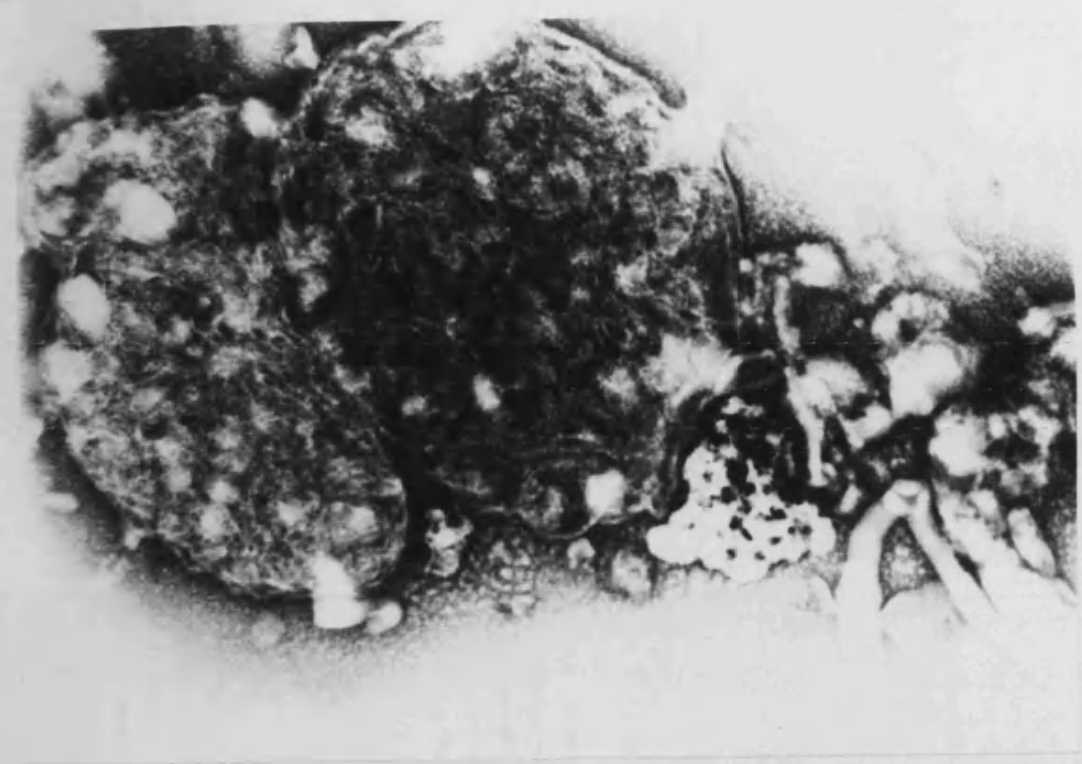


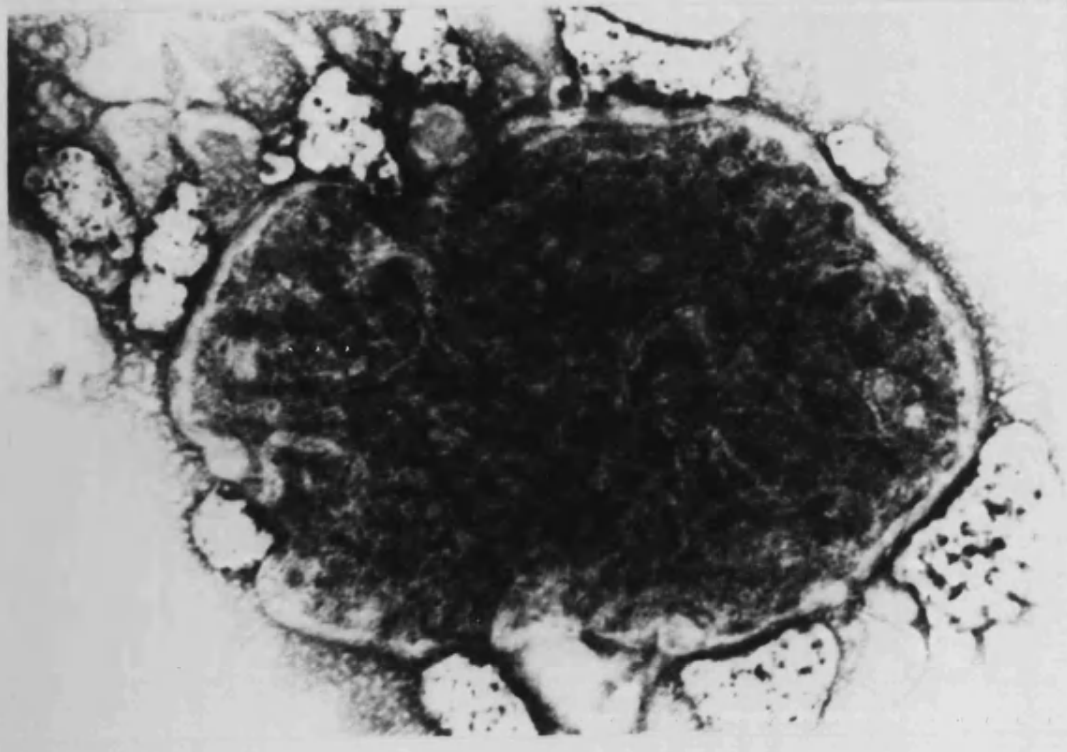
Fig.29 Electron micrographs of negatively stained rat striatal synaptosomes viewed at 33,000 x magnification. Scale bars denote actual sizes of structures.

a



0. I4μm

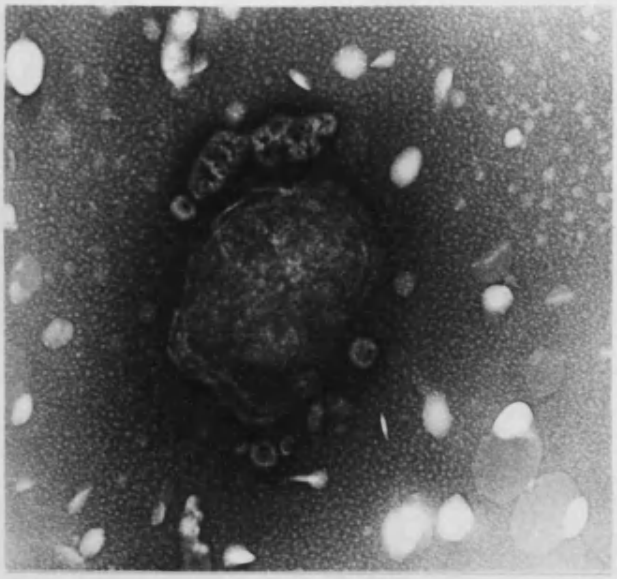
b



0. I4μm

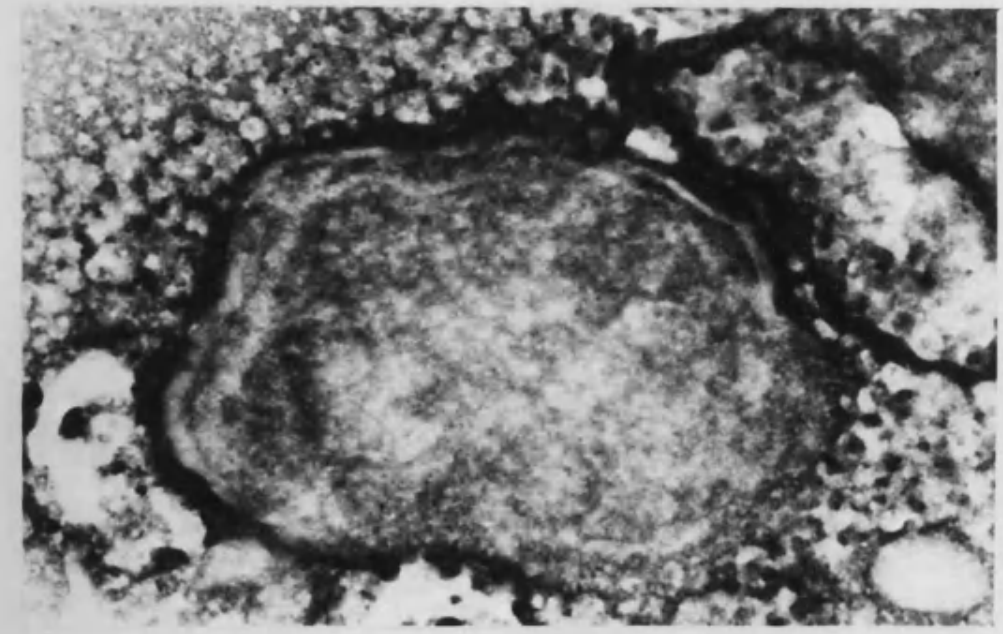
Fig.30 Electron micrographs of negatively stained locust ganglionic synaptosomes (H, 3.2.5) viewed at a) 33,000 x and b) 100,000 x magnification. Scale bars denote actual sizes of structures.

a



0.18μm

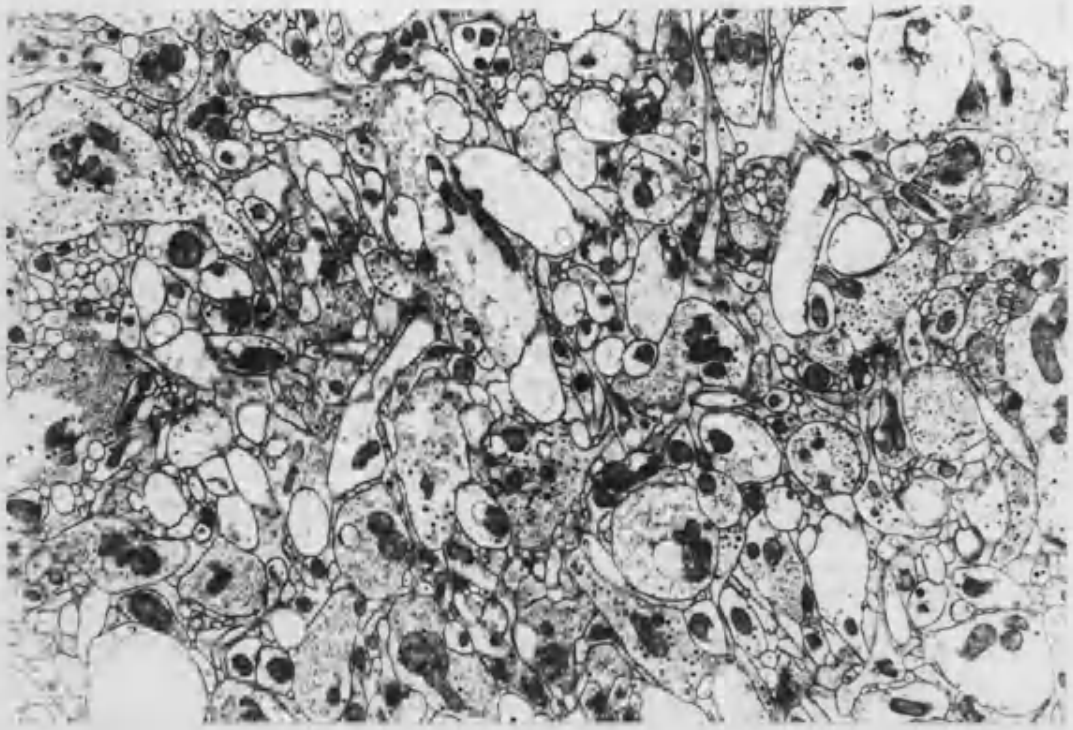
b



0.06μm

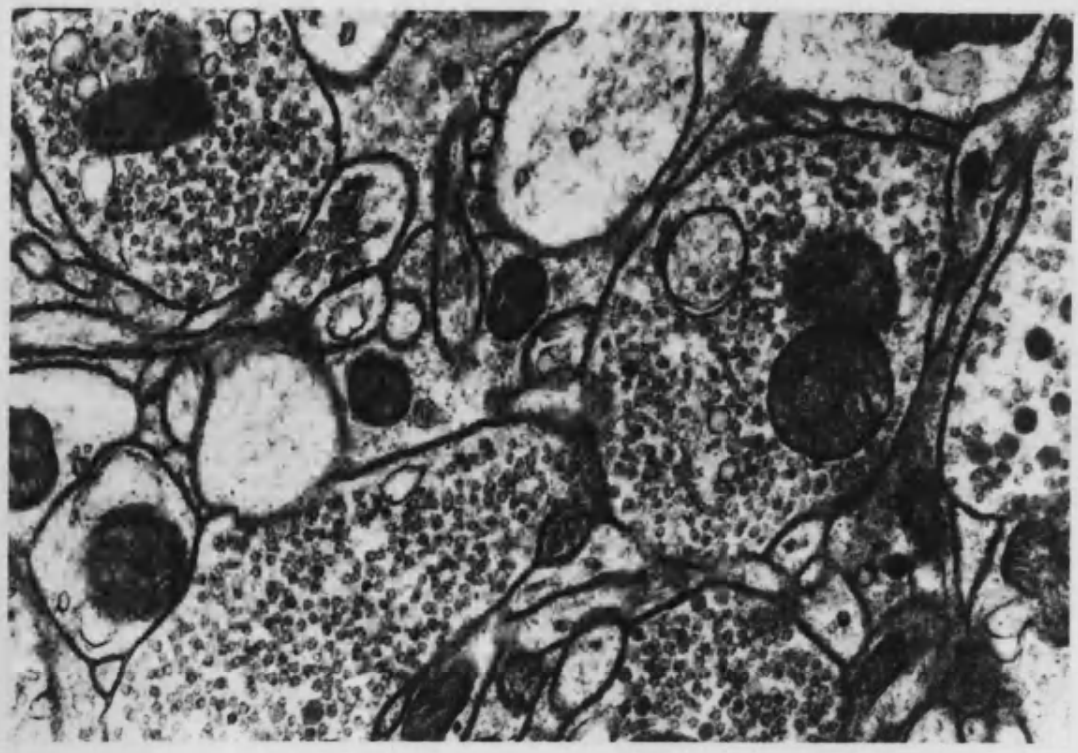
Fig.31 Transmission electron micrographs of sections through whole locust supraoesophageal ganglia viewed at a) 3,300 x and b) 33,000 x magnification. Scale bars denote actual sizes of structures.

a



I.48μm

b



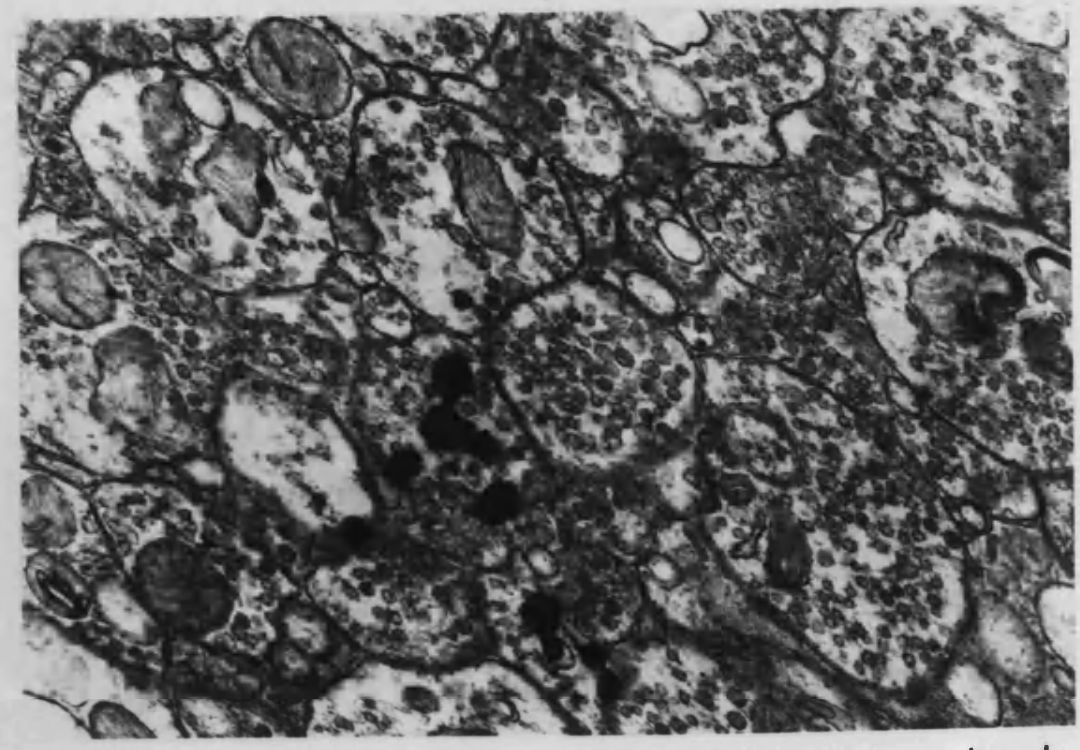
0.35μm

Fig.32 Transmission electron micrographs of sections through pelleted synaptosome-rich homogenate, H (3.2.5) viewed at a) 3,300 x and b) 33,000 x magnification. Scale bars denote actual sizes of structures.

a

1.40μm

b



0.36μm

3.3.2.2/ TRANSMISSION ELECTRON MICROSCOPY OF LOCUST SUPRAOESOPHAGEAL GANGLIA AND THE RESULTING SYNAPTOSOME-RICH HOMOGENATE, H

Sections of locust supraoesophageal ganglia and the homogenate, H, made from these ganglia (3.2.5) were visualized by transmission electron microscopy (3.2.7). Representative electron micrographs are shown in Figs.31&32.

3.3.3/ [^{3}H]Choline uptake by H and HF

In addition to a morphological monitor for synaptosomes, an assay was required which would give an indication of the physiological viability of the synaptosomes, prior to any investigation of GABA uptake by the preparation. Therefore the uptake of [^{3}H]choline was studied in H, and the filtered homogenate, HF, as described in 3.2.9. The homogenate accumulated [^{3}H]choline on incubation at 30^{o}C and the time course of this uptake is shown in Fig.33. The uptake following incubation at 30^{o}C for 30 min was reduced by over 95% by incubation on ice, in the presence of detergent, or in the presence of the mammalian [^{3}H]choline uptake inhibitor, hemicholinium-3 (Fig.34).

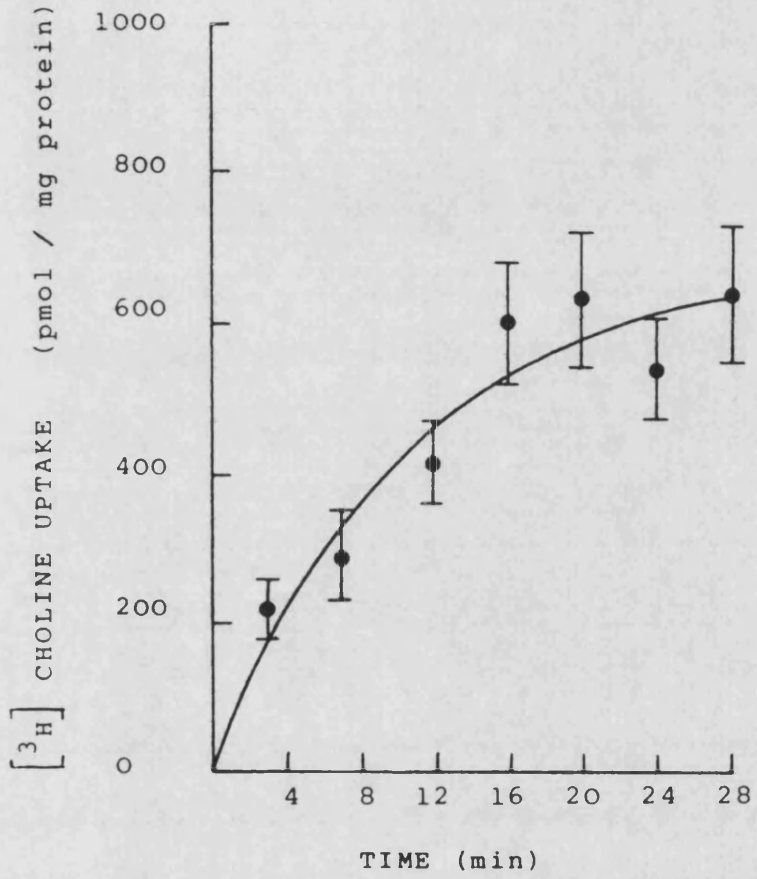


Fig.33 Time course of [^{3}H]choline uptake (7 μM) by the locust ganglionic homogenate, H (3.2.5) at 30^{o}C. Results are the means of four separate homogenates ±SEM.

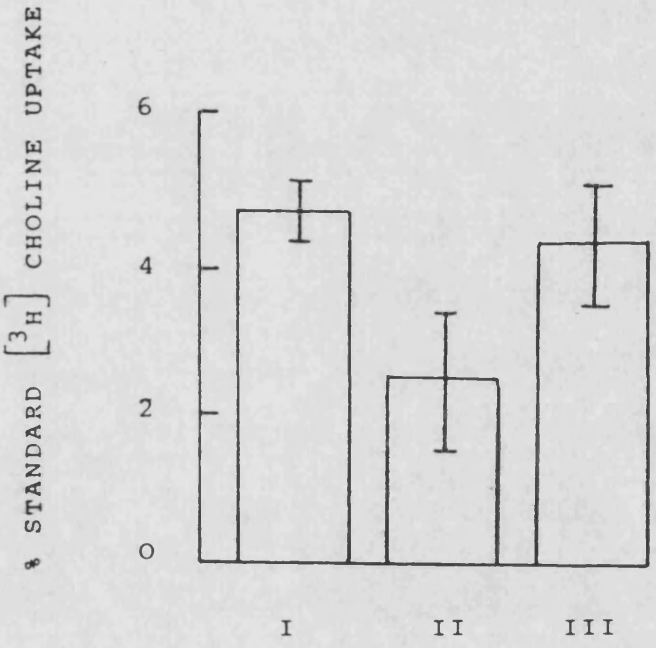


Fig.34 Effect of incubation on ice (I), in the presence of 1% (v/v) TritonX-100 (II) and in the presence of 0.1 mM hemicholinium-3 (III) on the uptake of [^{3}H]choline (7 μM) by H following a 30min incubation. Standard uptake is the uptake at 30^{o}C following a 30 min incubation. Results are the means of four separate homogenates ±SEM.

3.3.3.1/ STABILITY OF H ON STORAGE

The time course of [^{3}H]choline uptake was not altered after storage of H on ice for 1 h (Fig.35).

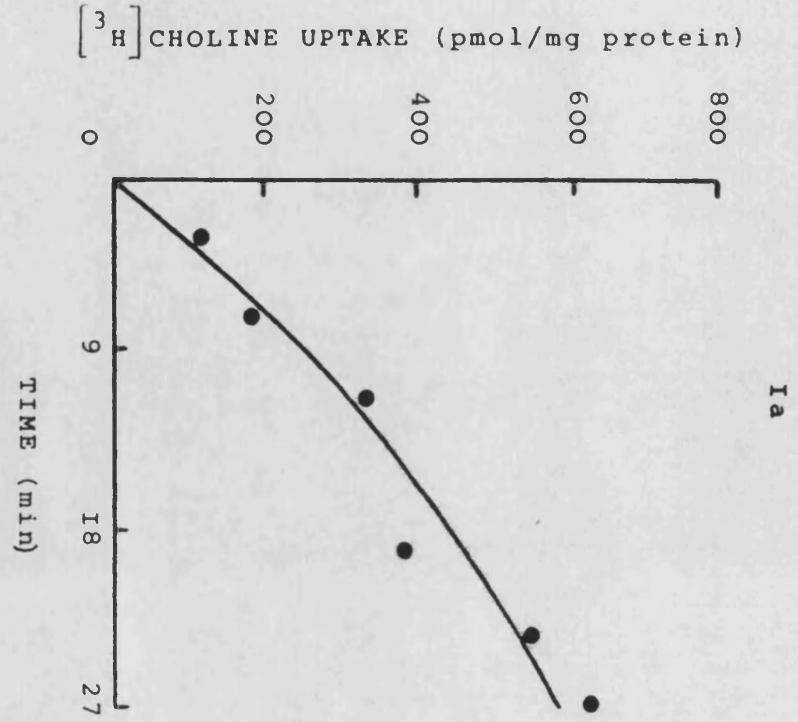
3.3.3.2/ EFFECT ON [^{3}H]CHOLINE UPTAKE OF FILTERING H

The effect on [^{3}H]choline uptake of filtering H was investigated in three separate homogenates (Fig.36). The uptake in HF was not significantly lower than that in H (II, Fig.36, by students t-test, $p > 0.1$), and the filtered homogenate, HF, was used in all subsequent investigations.

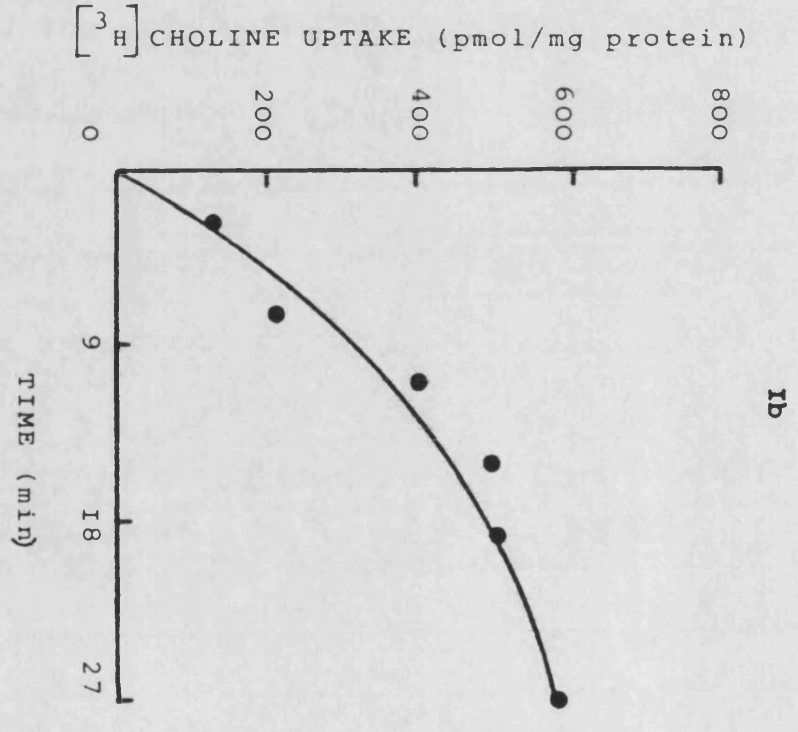
3.3.3.3/ EFFECT OF FILTER TYPE ON [^{3}H]CHOLINE UPTAKE BY HF

The uptake of [^{3}H]choline by HF was routinely terminated by filtration through Millipore 0.45 μm pore size cellulose nitrate filters. This uptake was compared with [^{3}H]choline uptake observed using glassfibre filters, boiled filters, filters with a larger pore size (0.65 μm) and filters supplied by Whatman (Fig.37). For economic reasons, Whatman 0.45 μm pore size cellulose nitrate filters (unboiled) were used in all subsequent assays because the uptake of [^{3}H]choline observed when using these filters was not significantly different from that observed when using the more expensive equivalent

Fig.35 Effect of storage of homogenate, H, on ice for 1 h (b) on the time course of [^{3}H]choline uptake (7 μM, 30°C) by two homogenates, I & II, compared with the uptake in the fresh homogenate (a).

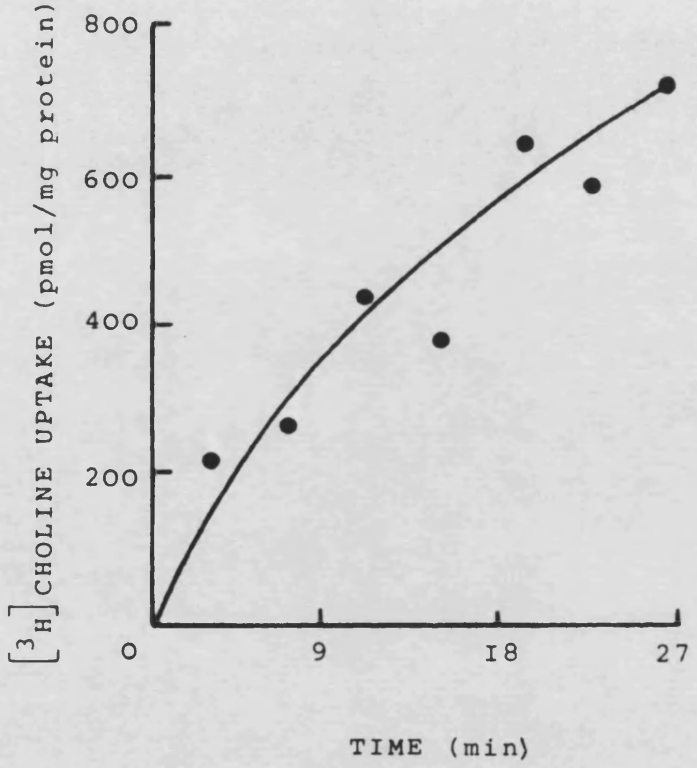

[3H] CHOLINE UPTAKE (pmol/mg protein)
0
200
400
600
800
TIME (min)
0
9
18
27

Ia

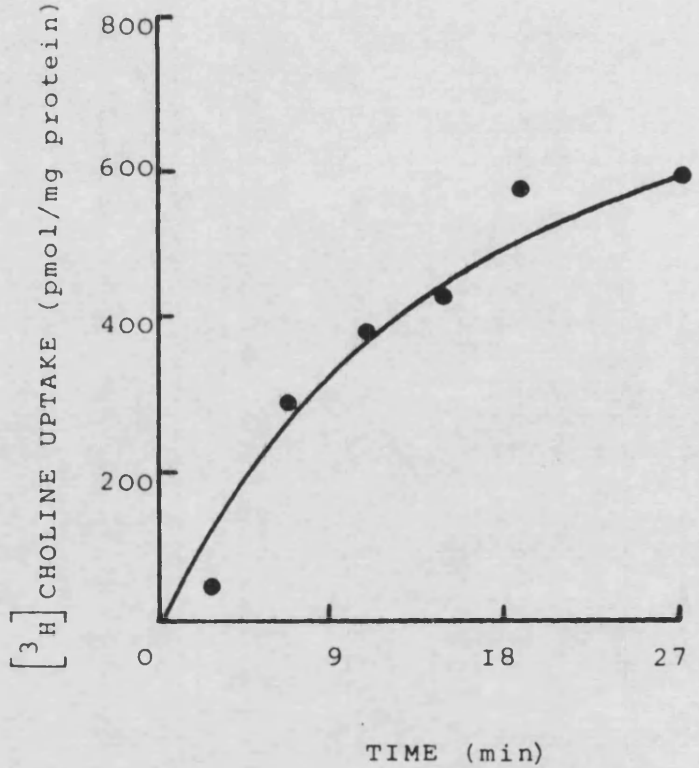

[3H] CHOLINE UPTAKE (pmol/mg protein)
0
200
400
600
800
TIME (min)
0
9
18
27

Ib

IIa


[3H] CHOLINE UPTAKE (pmol/mg protein)
800
600
400
200
0
9
18
27
TIME (min)

IIb


[3H] CHOLINE UPTAKE (pmol/mg protein)
800
600
400
200
0
9
18
27
TIME (min)

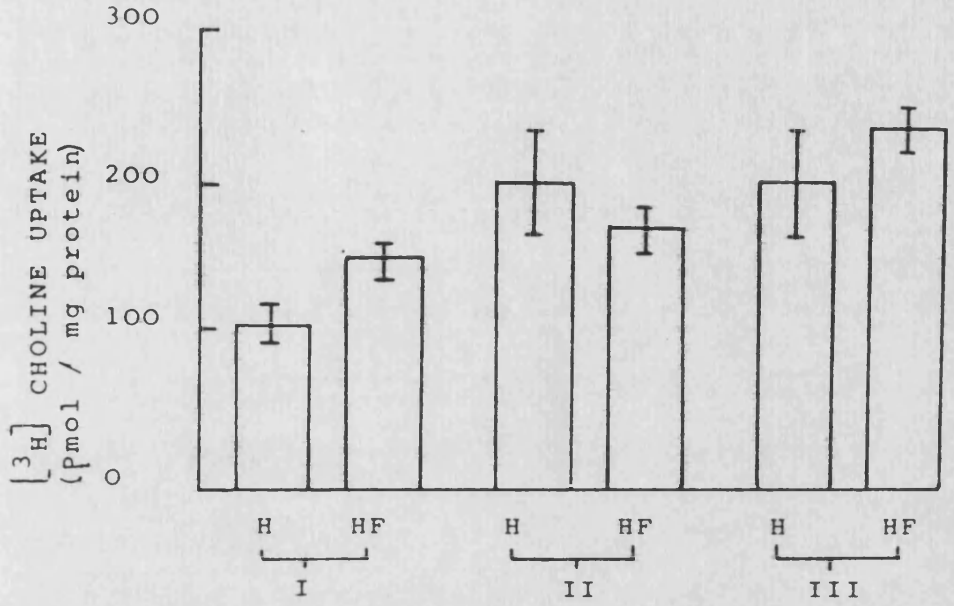
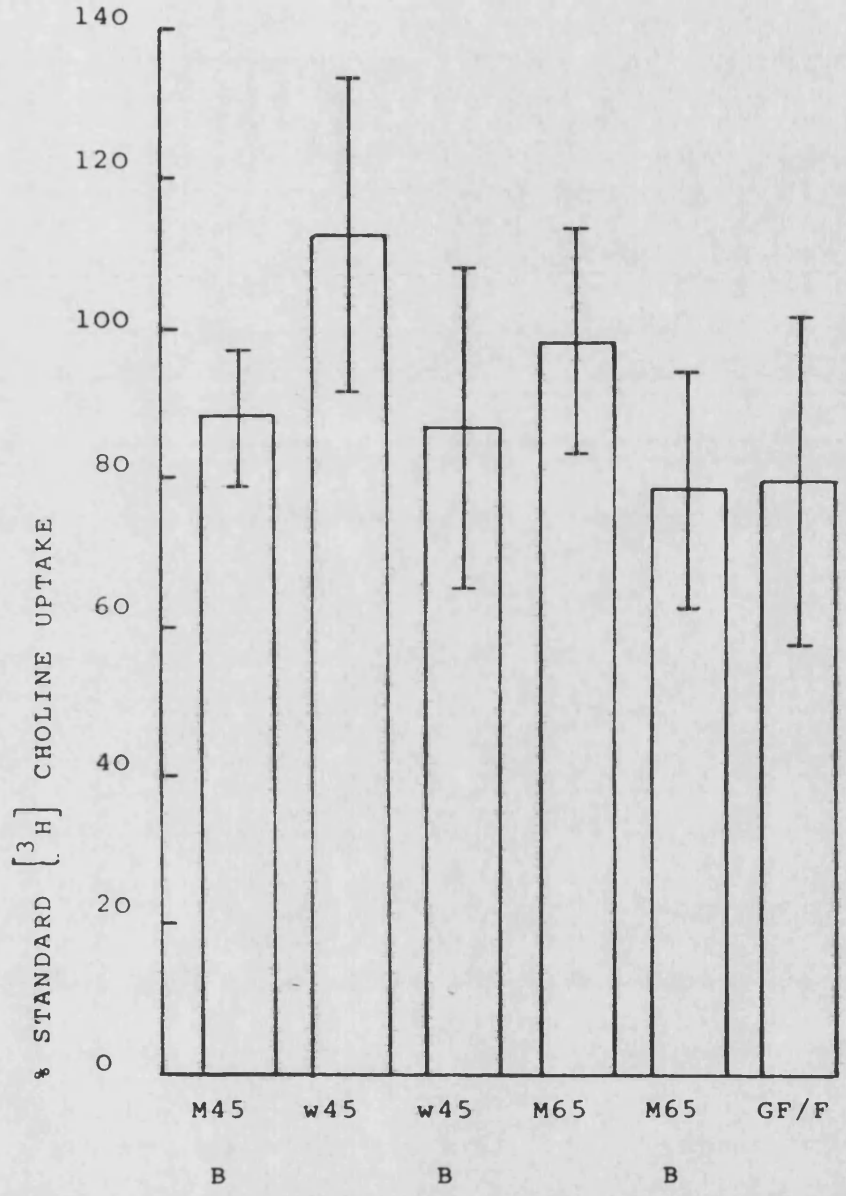


Fig.36 Effect of filtering the homogenate, H, to give HF, on [^{3}H]choline uptake. H was filtered through nylon bolting cloth (3.2.5) and both H and HF were incubated with 7 μM [^{3}H]choline for 20 min. Results are the means of six determinations ±SEM for three separate homogenates, I, II and III.

Fig.37 Effect of filter type on [^{3}H]choline uptake (7 μM, 20 min incubation) by HF. M, Millipore; w, Whatman; 45, 0.45 μm pore size cellulose nitrate; 65, 0.65 μm pore size cellulose nitrate; GF/F, Whatman GF/F glassfibre; B, boiled. Standard [^{3}H]choline uptake (100%) is the uptake observed using unboiled Millipore 0.45 μm pore size cellulose nitrate filters. Results are the means of at least six determinations ±SEM.


140
120
100
80
60
40
20
0
% STANDARD [3H] CHOLINE UPTAKE
M45
w45
w45
M65
M65
GF/F
B
B
B

filters from Millipore.

3.3.3.4/ EFFECT OF INCUBATION TEMPERATURE ON [^{3}H]CHOLINE UPTAKE BY HF

The effects of incubation temperature (20-70 ^{o}C) and hemicholinium-3 on [^{3}H]choline uptake were investigated in HF (Fig.38).

3.3.3.5/ EFFECT OF VARIOUS BUFFER TREATMENTS ON [^{3}H]CHOLINE UPTAKE BY HF

The effects of the omission of glucose, the addition of sodium azide and the gassing (95%/5%, O_2/CO_2) of the incubation buffer on [^{3}H]choline uptake were investigated in HF (Table 13).

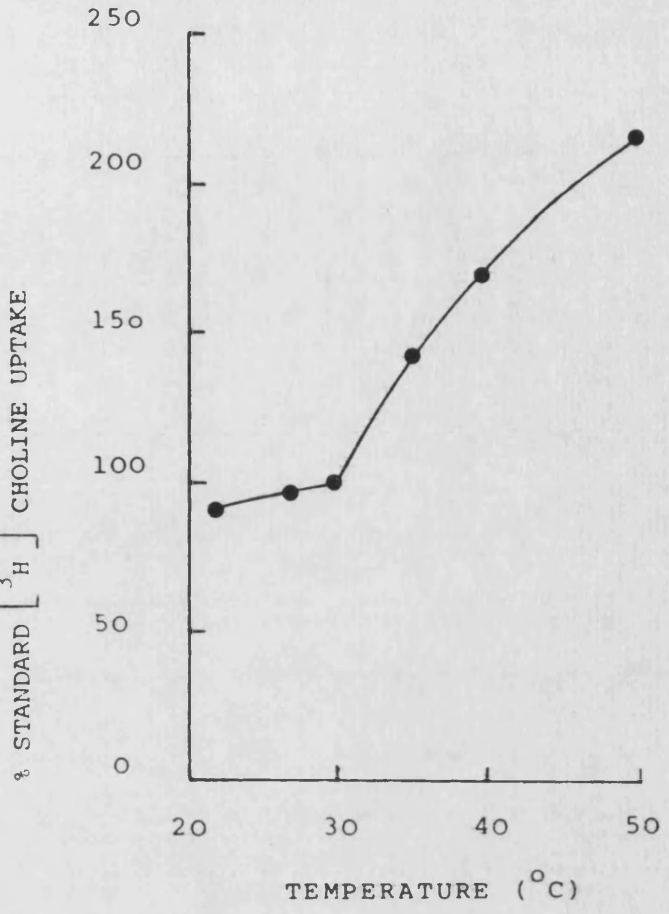
3.3.4/ Release of radioactivity from [^{3}H]choline-loaded HF

Samples of HF were preloaded with [^{3}H]choline and the release of radioactivity then compared in the presence and absence of 50 mM K^+ as described (3.2.10). Using triplicate assays, the radioactivity released in the presence of K^+ was 197.3 $\pm$ 22.4% of the radioactivity released in the absence of K^+ (mean of six experiments $\pm$SEM).

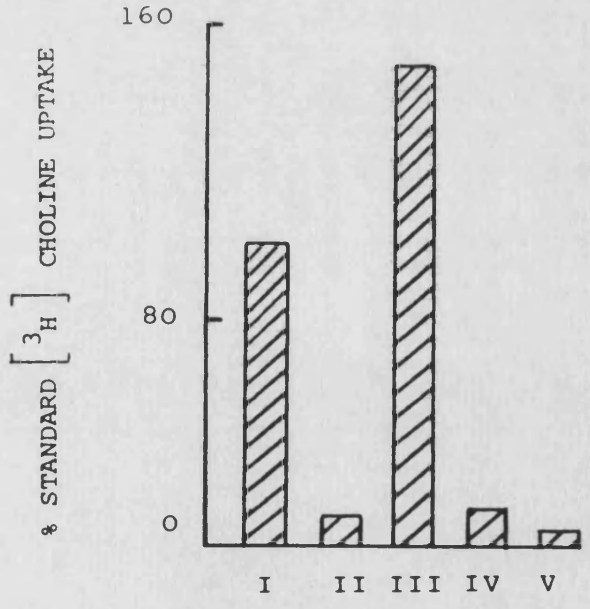
The supernatants from Na^+- and K^+-stimulated, [^{3}H]choline-loaded samples of HF were extracted as

Fig.38 a) Effect of incubation temperature on [^{3}H]choline uptake (7 μM, 30 min incubation). Results are from a single experiment using triplicate determinations. Similar results were obtained in a second experiment. b) Effect of temperature (I & II, 30°C; III & IV, 50°C; V, 70°C) and 0.1 mM hemicholinium-3 (II & IV) on [^{3}H]choline uptake (7 μM, 30 min incubation). Results are from a single experiment using triplicate determinations. In both a) and b) 100% standard [^{3}H]choline uptake is the uptake obtained with a 30°C incubation.

a.


250
200
150
100
50
0
% STANDARD [3H] CHOLINE UPTAKE
20
30
40
50
TEMPERATURE (°C)

b.


160
80
0
% STANDARD [3H] CHOLINE UPTAKE
I
II
III
IV
V

BUFFER TREATMENT	% STANDARD [^{3}H]CHOLINE UPTAKE
GLUCOSE OMISSION	122.5 ± 35.1
1 mM SODIUM AZIDE	97.2 ± 27.2
GASSED WITH (95%/5%) O_2/CO_2	149.6 ± 46.0

Table 13 Effect of various buffer treatments on [^{3}H]choline uptake (7 μM, 20 min incubation) by HF. Results are the means of six determinations ±SEM.

described (3.2.11) and the extracts analysed by tlc (3.2.12) for their Ch and ACh content. An example is shown of the radioactivity recovered from samples released from HF and from a sample of standard [^{3}H]choline, following extraction and tlc (Table 14).

3.3.4.1/ THIN LAYER CHROMATOGRAPHY OF CHOLINE SPECIES

Thin layer chromatograms gave consistent separation of standard choline species as judged by their R_f values (Table 15). The extracted samples were also well separated though their R_f values were more variable (Table 15) due to varying amounts of other residues affecting the mobilities of the samples.

On extraction of samples of supernatants of Na^+- and K^+-stimulated samples of [^{3}H]choline-loaded HF, radioactivity was consistently recovered from both Ch and ACh spots, irrespective of the stimulus, but the ratio of the radioactivity in the ACh spot to that in the Ch spot was always higher in the K^+-stimulated sample than in the Na^+-stimulated sample (Fig.39).

3.3.5/ [^{3}H]GABA uptake by HF

Having developed an assay for the uptake of [^{3}H]choline by HF, [^{3}H]GABA uptake studies could be performed on samples of HF which were known to be physiologically active by their capacity for [^{3}H]choline uptake. Hence the assays of [^{3}H]GABA uptake by HF

SAMPLE	% RECOVERY OF RADIOACTIVITY		
	K^+	Na^+	STANDARD
1^{st} EXTRACTION	47	44	32
2^{nd} EXTRACTION	12	7	11
TOTAL EXTRACTED	59	51	43
TOTAL TLC SCRAPINGS	7.7	5.2	1.3

Table 14 Example of the radioactivity recovered from extracted supernatants from K^+- and Na^+-stimulated, [^{3}H]choline-loaded HF (see 3.2.10,11), and a standard [^{3}H]choline sample. The 1^{st} and 2^{nd} extractions represent the first two extractions with heptanone prior to any extraction with HCl, and are summed to give 'TOTAL EXTRACTED'.

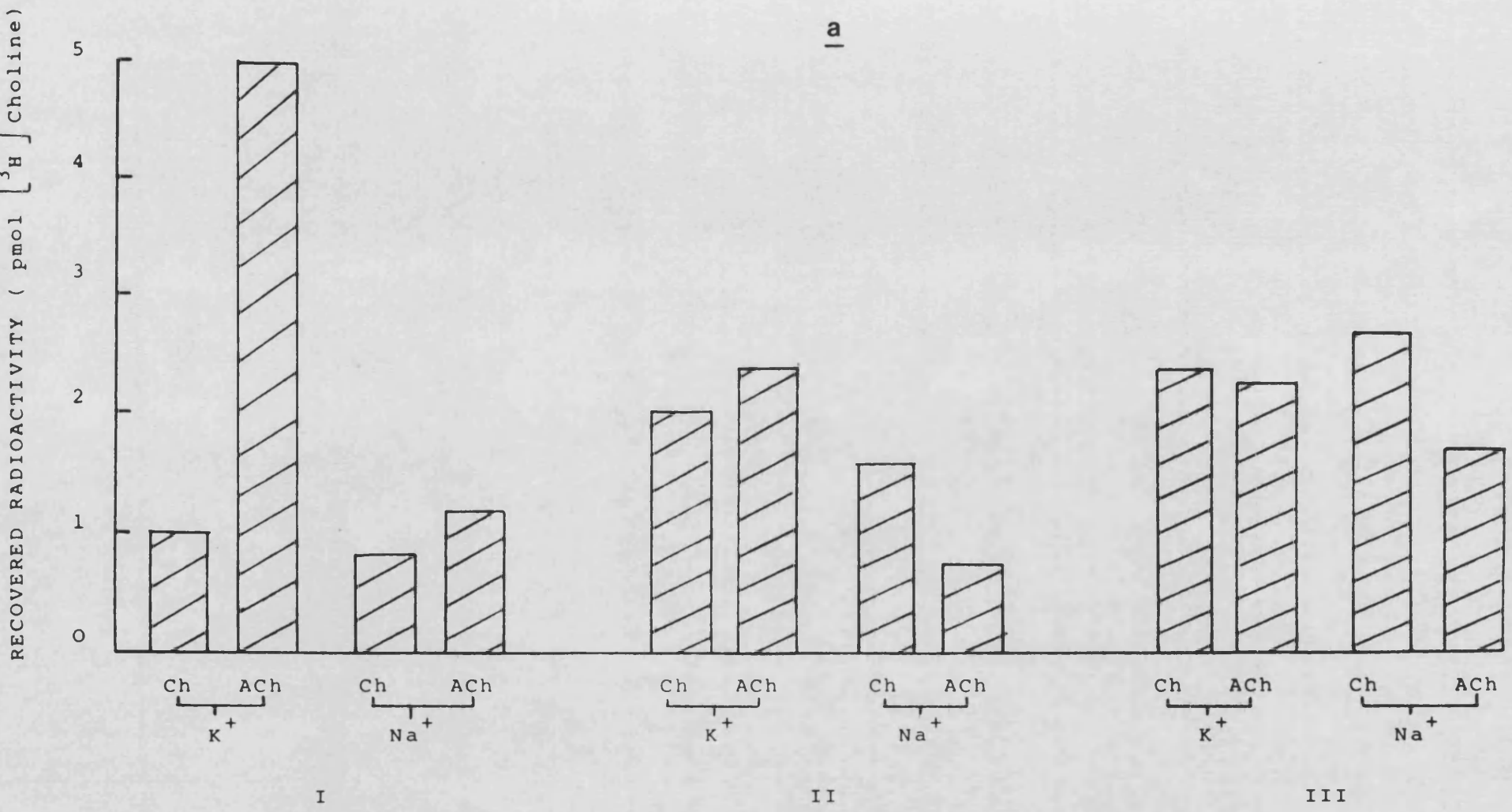
COMPOUND	R_f	
	STANDARD	EXTRACTED SAMPLE
CHOLINE	0.387 ± 0.004	0.28 - 0.36
ACETYL CHOLINE	0.477 ± 0.004	0.39 - 0.44
PROPYL CHOLINE	0.602 ± 0.003	0.51 - 0.58
BUTYRYL CHOLINE	0.737 ± 0.007	0.66 - 0.73

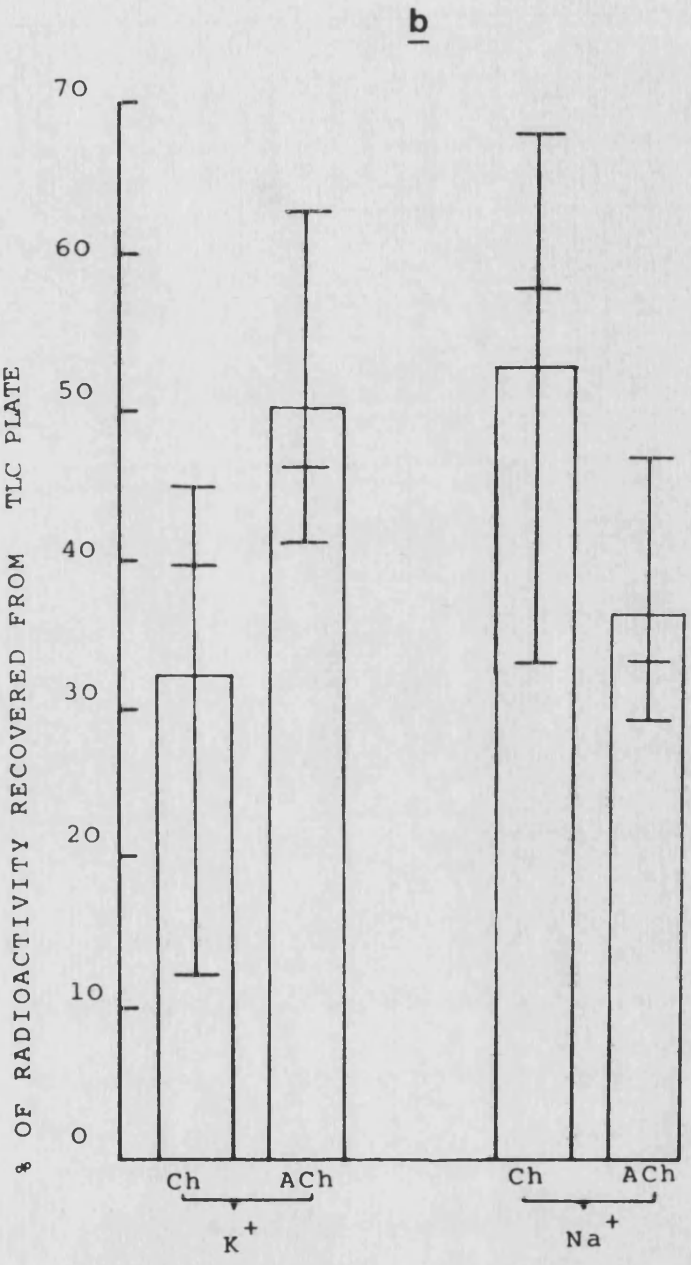
Table 15 The R_f values of standard and extracted species following tlc. Results are the means of six chromatograms ±SEM for the standards, and the range of values for six extracted samples.

Fig.39 Tlc of extracted samples of supernatants of [^{3}H]choline-loaded HF, stimulated either with K^+- or Na^+-buffer (see 3.2.11).

a) Radioactivity recovered in the choline spot (Ch) and acetylcholine spot (ACh) from three separate experiments, I, II and III. Results are expressed in terms of the [^{3}H]choline originally loaded.

b) The data from the three separate experiments in a) combined and expressed as means, in terms of the total radioactivity recovered from the tlc plate. Bars denote the results in the individual experiments.


a
RECOVERED RADIOACTIVITY (pmol [3H] Choline)
5
4
3
2
1
0
Ch
ACh
K+
Na+
I
II
III


b
70
60
50
40
30
20
10
0
% OF RADIOACTIVITY RECOVERED FROM TLC PLATE
Ch
ACh
Ch
ACh
K+
Na+

(3.2.13) were initially carried out in parallel with standard [^{3}H]choline uptake assays on the same preparation (results not shown). HF accumulated GABA on incubation at 30^{o}C and a time course of this uptake is shown in Fig.40. The uptake was greatly reduced by incubation on ice or in the presence of 50 mM K^+ (Fig.40).

3.3.5.1/ EFFECT OF GABA-T INHIBITORS ON [^{3}H]GABA UPTAKE BY HF

[^{3}H]GABA uptake was routinely assayed in the presence of the GABA transaminase inhibitor, aminooxyacetic acid (AOAA). The time course of [^{3}H]GABA uptake was therefore investigated in the presence of AOAA, in the absence of any GABA-T inhibitor and in the presence of a second GABA-T inhibitor, gabaculline (Fig.41).

3.3.5.2/ EFFECTS OF NIPECOTIC ACID AND 2,4-DABA ON THE TIME COURSE OF [^{3}H]GABA UPTAKE BY HF

The effects of the mammalian GABA uptake inhibitors, nipecotic acid and 2,4-diaminobutyric acid (2,4-DABA) on the time course of [^{3}H]GABA uptake by HF were studied in two separate homogenates (Fig.42).

Fig.40 Time course of [^{3}H]GABA uptake (0.1 μM) by HF, in the presence of 50 μM aminooxyacetic acid, incubated at 30°C (●), on ice (O) and in the presence of 50 mM K^{+} (▲). Data are from a single experiment but similar data were obtained in a second experiment. The broken line indicates a possible biphasic uptake profile.

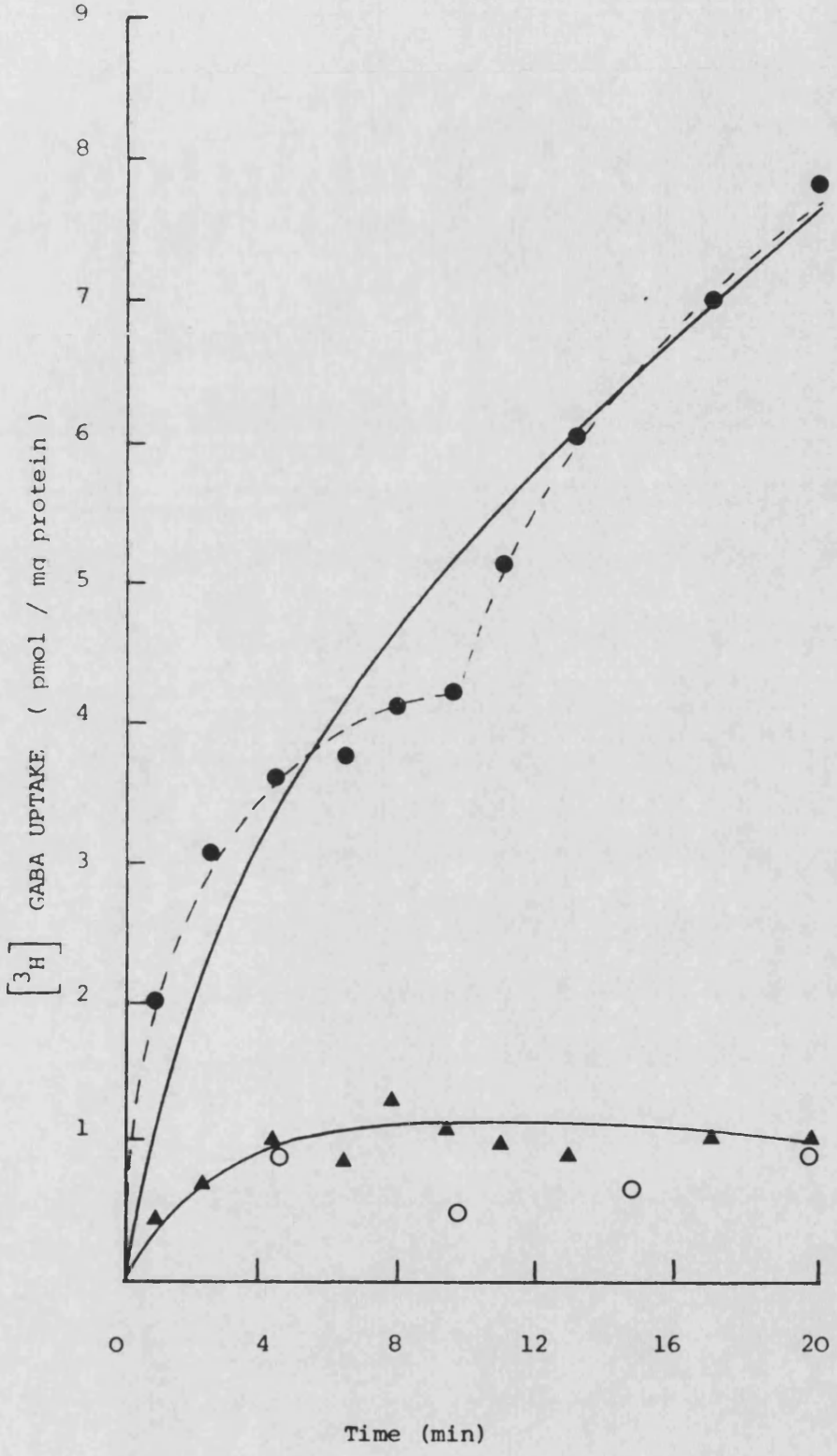
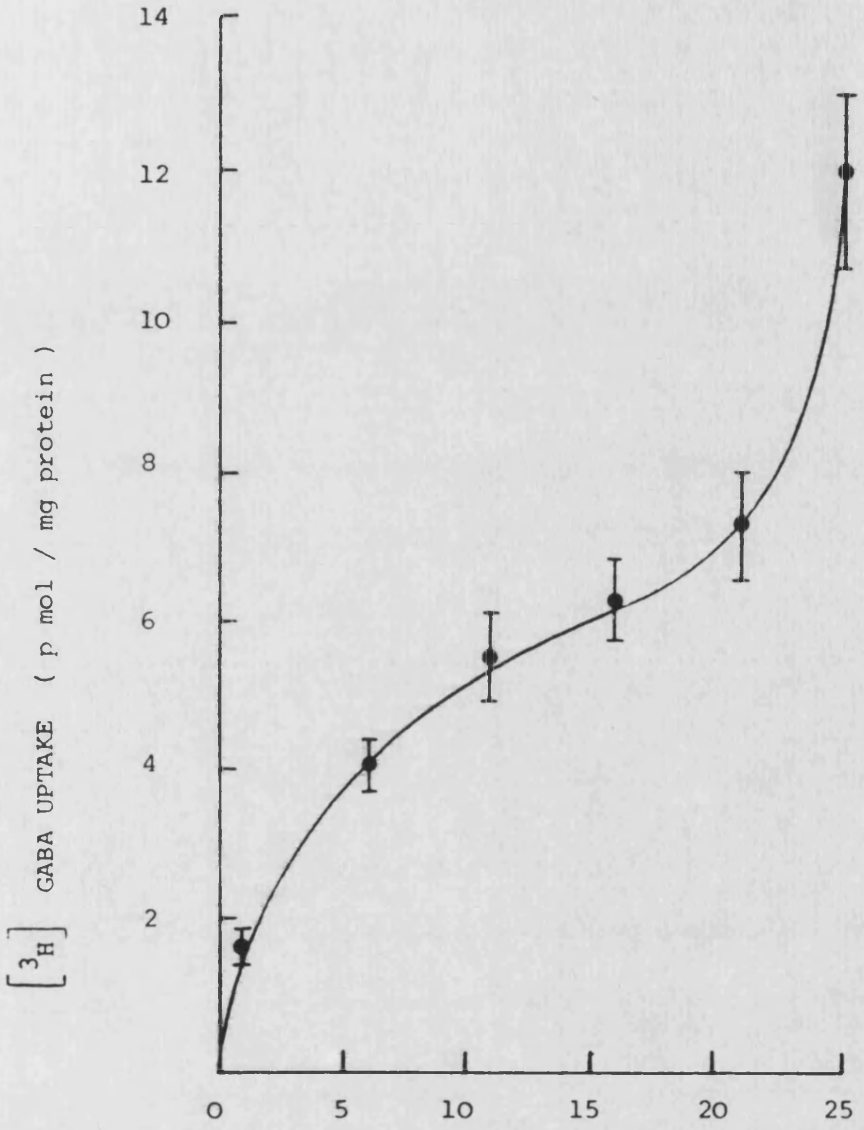
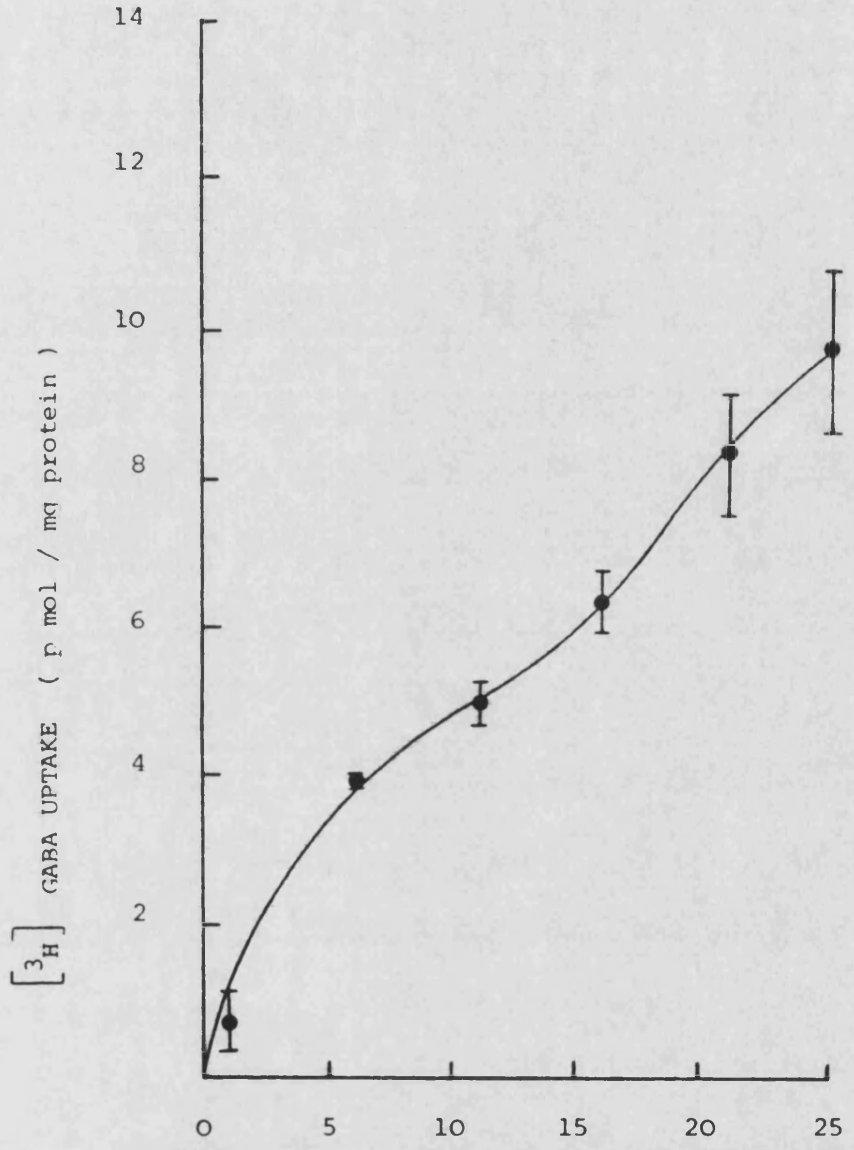

[3H] GABA UPTAKE (pmol / mg protein)
9
8
7
6
5
4
3
2
1
0
4
8
12
16
20
Time (min)

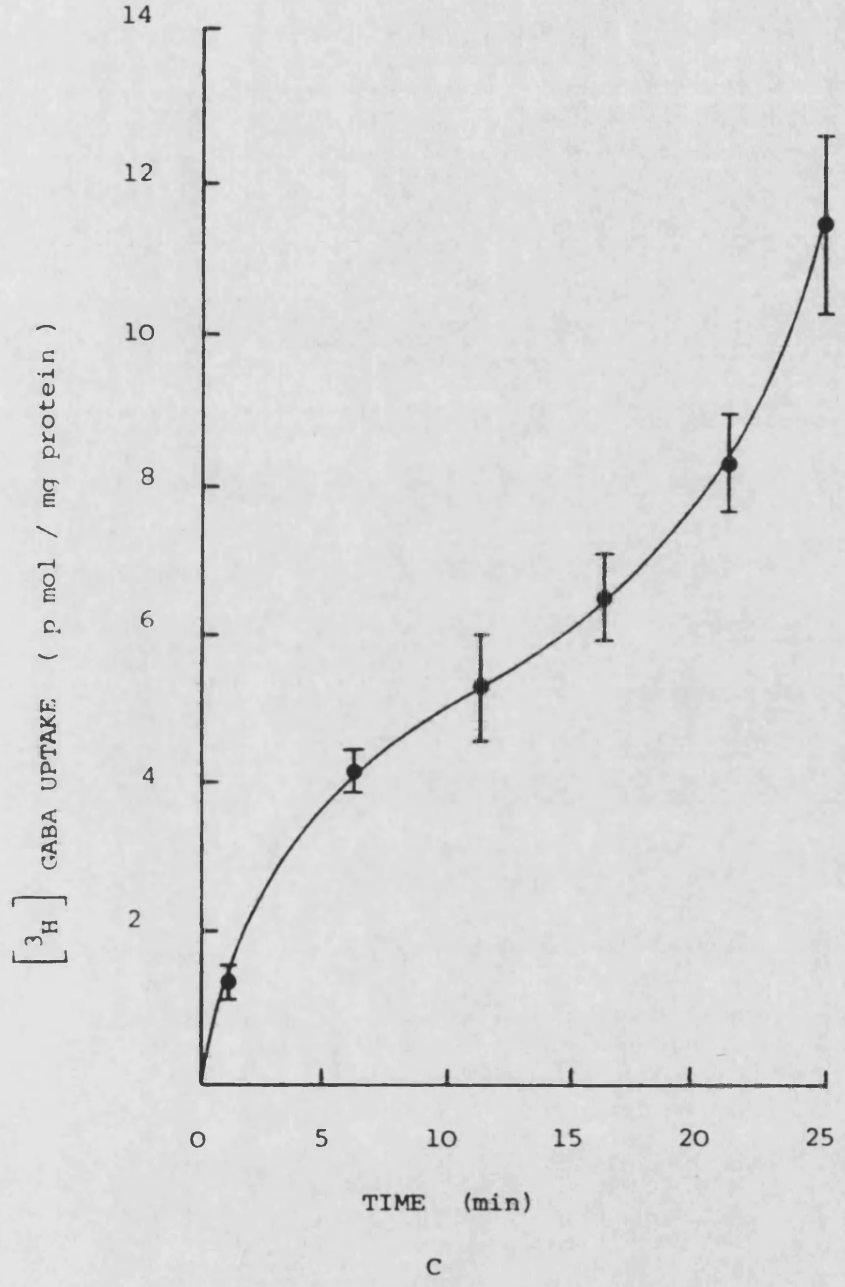
Fig.41 Effects of inhibitors of GABA-T on the time course of [^{3}H]GABA uptake (0.1 μM) by HF. A, 50 μM aminooxyacetic acid; B, no inhibitor; C, 10 μM gabaculline. Results are the means of four separate experiments ±SEM.


14
12
10
8
6
4
2
[3H] GABA UPTAKE (p mol / mg protein)
0
5
10
15
20
25
TIME (min)

A

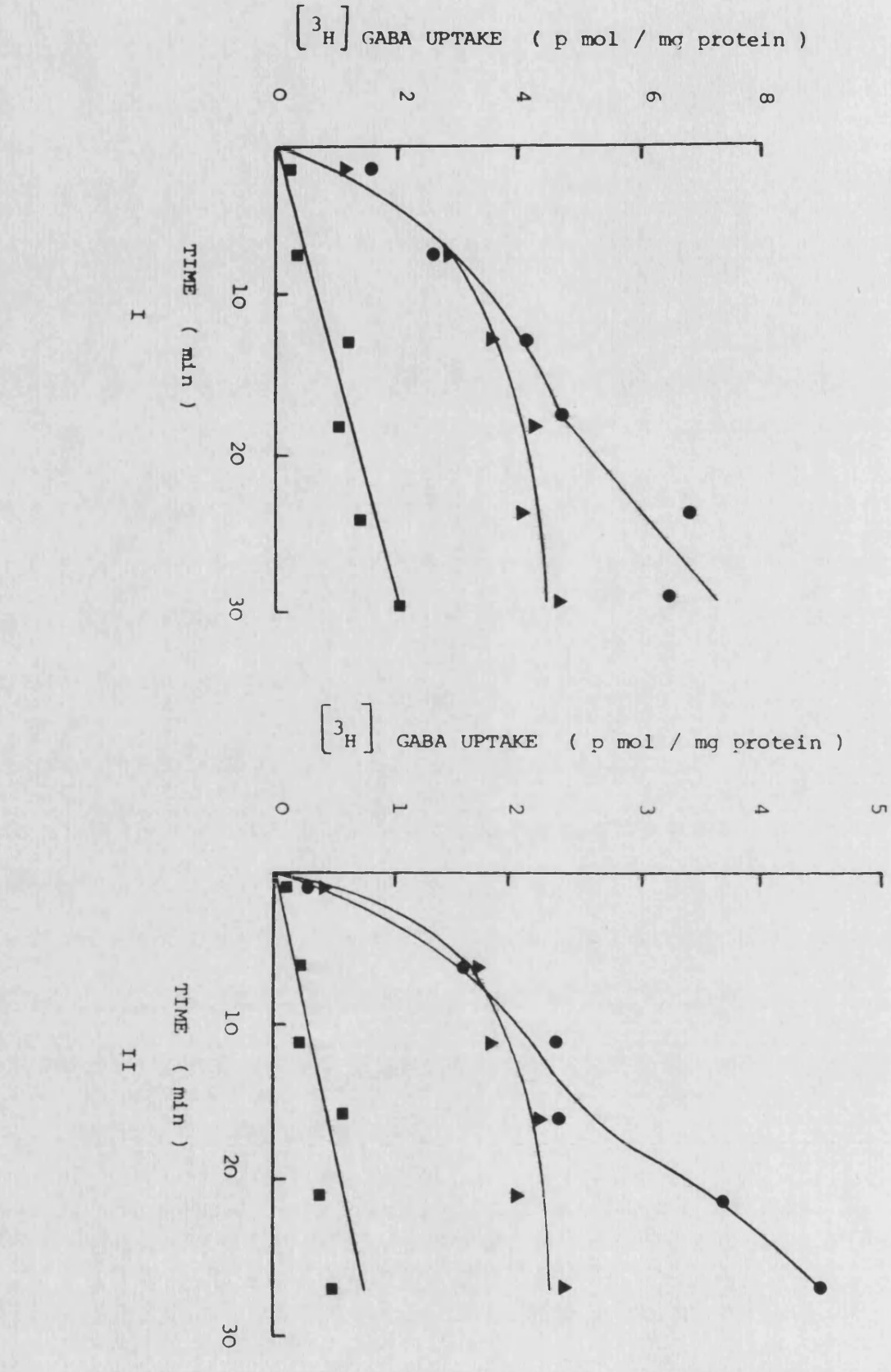

14
12
10
8
6
4
2
[3H] GABA UPTAKE (p mol / mg protein)
0
5
10
15
20
25
TIME (min)

B


14
12
10
8
6
4
2
[3H] GABA UPTAKE (p mol / mg protein)
0
5
10
15
20
25
TIME (min)

C

Fig.42 Effect of 0.1 mM nipecotic acid (▲) and 0.1 mM 2,4-DABA (■) on the standard time course (●) of [^{3}H]GABA uptake (0.1 μM) by HF in two separate homogenates, I & II.


[3H] GABA UPTAKE (p mol / mg protein)
0
2
4
6
8
10
20
30
TIME (min)
I
[3H] GABA UPTAKE (p mol / mg protein)
0
1
2
3
4
5
10
20
30
TIME (min)
II

3.3.6/ Release of radioactivity from [^{3}H]GABA-loaded HF

Following the loading of samples of HF with [^{3}H]GABA they were incubated with Na^+-, K^+- or K^+Ca^{2+}-buffers (Table 9, 3.2.14) and the radioactivity released was then measured either by a centrifugation (3.2.14a) or a filtration method (3.2.14b). The results using the two methods are compared in Table 16a. The release measured by the filtration method is expressed in Table 16b having assumed the Na^+-stimulated release as the background release.

a

METHOD	RADIOACTIVITY RELEASED (% OF TOTAL LOADED)		
	Na^+	K^+Ca^{2+}	K^+
CENTRIFUGATION	72.3 ± 2.6	77.7 ± 2.7	73.4 ± 3.7
FILTRATION	34.0 ± 2.8	39.2 ± 3.0	34.9 ± 2.8

b

STIMULATION	RADIOACTIVITY RELEASED (Na^+ STIMULATION AS) (BACKGROUND)
K^+ Ca^{2+}	5.2 ± 0.6
K^+	0.9 ± 1.6

Table 16 Release of radioactivity from [^{3}H]GABA-loaded HF by different buffers (see 3.2.14).
a) Comparison of centrifugation and filtration methods. Results are expressed as a % of the total loaded radioactivity and are the means of five or six separate determinations ±SEM.
b) Release measured by the filtration method and expressed as a value with the quantity of radioactivity released by Na^+-buffer subtracted as a background. Results are the means of six separate determinations ±SEM.

3.4/ DISCUSSION

3.4.1/ The use of enzymes to screen for intact synaptosomes

Initial studies were aimed at identifying an enzyme which could be used to indicate the possible presence of synaptosomes in a preparation, by the presence of occluded cytoplasm. To be of use, an enzyme must have a purely cytoplasmic location, be assayable under the synaptosomal buffer regime and have sufficient activity to be readily detected in a final preparation.

To these ends, lactate dehydrogenase (LDH), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and malate dehydogenase (MDH) were assayed in homogenates of locust supraoesophageal ganglia (Table 10). MDH exhibited over 35-fold greater activity than either of the other two enzymes making it the enzyme of choice on these grounds, especially as over 40% of this activity is still present when assayed in synaptosome buffer (results not shown). However, this activity was determined in a preparation which had undergone very harsh homogenization and which should therefore not have any residual occluded cytoplasm, yet 25% of the total MDH activity was still occluded. This activity is probably mitochondrial, as MDH is known to be present in both the cytosol and mitochondria (Lehninger, 1975), and would confuse any determination of the synaptosomal content of a preparation. The only possibility might be

to assay a mitochondrial enzyme (such as succinate dehydrogenase, Muller et al., 1968) in addition to MDH, in the presence and absence of TritonX-100, such that one could calculate the %occluded MDH activity which is non-mitochondrial by a ratios method.

GAPDH was the next best candidate on the grounds of total activity in the ganglion. However, the assay buffer for this enzyme is quite different from the buffer in which synaptosomes would be prepared and so the enzyme activity was compared under the two different buffer conditions (Table 11). The addition of 100 mM sucrose had little effect on GAPDH activity, but the reduction of pH from 7.6 to 7.4 resulted in 20% loss of activity. Furthermore, the use of phosphate/carbonate buffer (pH 7.4) rather than Tris-HCl buffer (pH 7.6) caused approximately 40% loss of activity. Despite these losses in enzyme activity, GAPDH activity would still be preferable to LDH; one would have to lose over 70% of the GAPDH activity before it was lower than the activity of LDH. However, the major problem with the assay of GAPDH is that it relies on the presence of arsenate; arsenate replaces phosphate in reacting with glyceraldehyde 3-phosphate and drives the reaction in the direction of what would normally be glyceraldehyde 1,3-diphosphate (and NADH production). Removal of the arsenate virtually abolishes the observed enzyme activity, and there is no means of determining the effect of arsenate on the biochemical viability of

insect synaptosomes. Therefore one cannot safely use an enzyme assay which has arsenate present, though an alternative would be to apply an enzyme-coupled assay for GAPDH (Wu & Racker, 1959).

There are other potential enzyme candidates (eg: triose phosphate isomerase) which could be investigated, but LDH, though present in low activity, does satisfy the requirements of an enzyme for screening for membrane-enclosed cytosol. Any further search for high-activity, cytosolic enzymes, which could be conveniently assayed, did not warrant the time required.

3.4.2/ The preparation of synaptosomes by a microscale floatation procedure

Initial attempts to isolate synaptosomes from locust ganglia followed the method of Breer & Jeserich (1980) whereby, following fractionation of the homogenate of nervous tissue, the synaptosomes are floated from sedimenting mitochondria by continuous Ficoll density gradient centrifugation. The resulting fractions were monitored for occluded LDH activity (Table 12). The LDH activity of the homogenate was routinely 50% occluded, but only 40% of the total activity of the homogenate was present in the fraction (P_2) which underwent the final centrifugation. Of this only 17% was occluded, and hence the bulk of the LDH activity recovered from the Ficoll gradient was in the supernatant rather than the pellicle or the pellet. It

should be noted that the measurements of LDH activity in fractions from the Ficoll gradient are prone to large errors owing to the very low levels of enzyme activity which are present. Certainly a pellicle was never obtained which was enriched in LDH activity as reported by Breer & Jeserich (1980). Whether or not the Ficoll floatation method is of use in purifying synaptosomes cannot be ascertained because the Ficoll gradient was never loaded with a fraction of high LDH activity, of which a high percentage was occluded. Indeed the basic problem in these experiments is probably two-fold. The initial homogenate may not possess a high content of synaptosomes, and any synaptosomes which are present do not survive the lengthy manipulations which follow.

3.4.3/ The production of a synaptosomal preparation from locust ganglia

Following the lack of success with the method of Breer & Jeserich (1980) in producing synaptosomes it was decided to start with the homogenate and develop a procedure for synaptosome production by monitoring each stage morphologically. The standard techniques of transmission electron microscopy are lengthy, requiring at least six days between the initial preparation of the sample and its visualization under the electron beam. Therefore a negative staining technique (Brenner & Horne, 1959) was developed, using rat striatal synaptosomes as a standard. The technique was adapted

for use with mammalian brain fractions by Horne & Whittaker (1962) but has not had extensive use, largely due to its poor delineation of ultrastructural detail (Jones, 1971, 1972). However, the technique is of use for the rapid evaluation of subcellular fractions (the sample can be visualized under the electron microscope within 30 min of the sample preparation). A further drawback with negative staining was that most of the stains used (such as phosphotungstate and lithium tungstate) damaged membrane systems by hypotonic disruption in the absence of prior fixation, such that the method gave highly variable results when used to visualize synaptosomes. However Muscatello & Horne (1968) reported that one could negatively stain membraneous structures with isotonic ammonium molybdate without prior fixation and this was successfully applied to the visualization of octopus synaptosomes by Jones (1971). On application of the technique to rat striatal synaptosomes (Fig.29) synaptosomes were visualized as membrane-enclosed structures of 0.6-1.2 μm diameter. Their size and appearance is consistent with the characteristics of negatively stained synaptosomes from other brain regions (Jones, 1975). The mottled appearance of the synaptosomes may be due to either synaptic vesicles or endoplasmic reticulum and the white profiles are probably an artefact caused by the sucrose in the preparation (Muscatello & Horne, 1968; Jones, 1975).

Armed with the negative staining screen for synaptosomal profiles, the locust supraoesophageal ganglia could be fractionated and studied under the electron microscope. To obtain a more gentle disruption of the ganglia, a teflon pestle was made for the microhomogenizer and the resulting homogenate was negatively stained. Synaptosomal profiles were visualized (Fig.30) which were reminiscent of those obtained with the control rat synaptosomes (Fig.29). The only difference was that the locust synaptosomes were smaller (0.4-0.6 μm dimeter) than their mammalian counterparts, but within the range of dimensions published for synaptosomes from other sources (0.2-5 μm, Jones, 1975). This homogenate was studied further by conventional transmission electron microscopy. By this method large numbers of synaptosomal structures were visualized (Fig.32) which were comparable with the nerve terminals in the intact ganglion (Fig.31), both in their variable dimensions (0.4-1.5 μm diameter) and heterogeneous content of synaptic vesicles. In an autoradiographic study of synaptosomes from rat cerebral cortex, Iversen & Bloom (1972) have reported that synaptosomes labelled with [^{3}H]GABA were 0.6 μm in diameter while unlabelled synaptosomes had a diameter of 0.35 μm, both of which are in the range of dimensions obtained with locust synaptosomes. Previous studies of insect synaptosomes have reported dimensions of 0.7-2 μm (Breer Jeserich, 1980; Breer, 1981a, 1982a; Gordon et

al., 1982; Dwivedy, 1985).

It was concluded from the electron microscopic studies that the homogenate was rich in synaptosomal profiles. However, Bradford et al. (1975) have demonstrated that morphological integrity is not necessarily a good index of biochemical viability of synaptosomes. Though the aim of the study reported in this thesis was to investigate GABA uptake, this is a poorly understood system in insect CNS. However, insect CNS is known to have a high density of cholinergic neurones (Breer, 1981b) and choline uptake has been characterized to a greater extent than GABA uptake in insect synaptosomes (Breer, 1982b). Therefore by studying [^{3}H]choline uptake in this synaptosome-rich homogenate, it was hoped that the biochemical viability of the synaptosomes could be demonstrated, and furthermore the [^{3}H]choline uptake could serve as a tool with which to characterize the optimal physical regime for the synaptosomes.

3.4.4/ Uptake of [^{3}H]choline by the synaptosome-rich homogenate, H

Mammalian brain possesses high and low affinity uptake systems for choline (Yamamura & Snyder, 1972, 1973; Simon & Kuhar, 1976). The two systems have affinity constants of 0.5-6 μM and >20 μM and have been extensively reviewed (Kuhar & Murin, 1978; Jope, 1979; Gibson & Blass, 1982). Most of the choline transported

by the high affinity system is converted to acetylcholine (Mulder et al., 1974), though there is evidence that acetylcholine can also be formed to a lesser extent from choline transported by the low affinity transporter (Mulder et al., 1974; Carroll & Goldberg, 1975). More recently though, Meyer et al. (1982) have reported that acetylation of choline only occurs after high affinity transport into rat cortical synaptosomes, while phosphorylation occurs after both high and low affinity transport. The high affinity uptake system occurs principally on cholinergic nerve terminals and is inhibited by hemicholinium-3 (HC-3) while the low affinity system is relatively insensitive to HC-3 (Yamamura & Snyder, 1973). High affinity choline transport is thought to be the regulating step in ACh synthesis in vertebrate brain (Jope, 1979).

When the synaptosome-rich homogenate, H, was incubated with 7 μM [^{3}H]choline at 30^{o}C it accumulated up to 600 pmol [^{3}H]choline/mg protein over a 30 min time course (Fig.33). This uptake was virtually abolished by incubation on ice (as one would expect for an active transport process), by 1% (v/v) TritonX-100 (as one would expect for uptake into a membrane-enclosed structure) and by HC-3 (which inhibits high affinity choline uptake in mammalian brain). The uptake reported here is comparable with the more pure preparation of synaptosomes from Locusta migratoria (Breer, 1982b) which, on incubation at 30^{o}C with 1 μM [^{3}H]choline,

accumulated 150 pmol [^{3}H]choline/mg protein in a linear manner over a 10 min time course. The uptake of 0.5 μM [^{3}H]choline by a synaptosome-enriched homogenate from rat brain began to saturate after a 12 min incubation at 30^{o}C (Yamamura & Snyder, 1973). However, in contrast to this, Dwivedy (1985) has reported that synaptosomes from the cockroach, Periplaneta americana, only accumulated 20 pmol [^{3}H]choline/mg protein over a 1-3 min time course, after which time the uptake declined. The cockroach synaptosomes were incubated with 1 μM [^{3}H]choline, and the only major difference in this study was that the synaptosomes were incubated at the lower temperature of 21^{o}C. The uptake of [^{3}H]choline at 22^{o}C by the homogenate described here, though, was very similar to that observed at 30^{o}C (Fig.38). Therefore it is difficult to understand the low, unstable uptake reported by Dwivedy (1985) unless the synaptosome preparation is very unstable. Evidently the homogenate, H, possesses a large [^{3}H]choline uptake capacity which has, at least in part, the characteristics of the classical mammalian high affinity choline uptake system.

This uptake capacity was used to further characterize the homogenate. Storage on ice for up to 1 hour (Fig.35) did not affect the uptake capacity of the homogenate, reflecting a certain degree of stability in the synaptosomal component of the preparation. Indeed an advantage of using the homogenate is its rapid preparation time which might result in greater stability

than purified insect synaptosomes which take 2-3 hours to prepare (Dwivedy, 1985). Because the preparation is a crude homogenate, it will contain pieces of undisrupted tissue which would be expected, by analogy with mammalian brain preparations, to act as the equivalent of brain slices. Wood & Sidhu (1986) have recently pointed out that brain slices and synaptosomes behave differently in uptake studies, owing to such considerations as ease of access of the ligand to the sites of uptake. Therefore the homogenate was filtered through nylon bolting cloth of 190 μm mesh. Although this might not eliminate small tissue aggregates, it should filter out large fragments of unhomogenized tissue. Such filtration had no effect on the specific [^{3}H]choline uptake capacity of the homogenate (Fig.36). This was not analysed any further (by kinetic analysis) as it was only the [^{3}H]choline uptake signal which was important and the filtered homogenate would be expected to be more homogeneous than the raw homogenate.

It has been reported that the type of filter used in the termination of the uptake of [^{14}C]glutamate by rat brain synaptosomes can affect the size of the signal (Wheeler, 1978). Also some manufacturers use detergent during the production of the filters (Dunbar, personal communication) and any residual detergent, which can be removed by boiling of the filters prior to their use, would affect the uptake signal. Therefore a range of filters, boiled and unboiled, was examined in

terminating the [^{3}H]choline uptake assay (Fig.37). The [^{3}H]choline uptake signal was not significantly affected by the use of different filters (though, if anything, boiled filters gave a reduced signal compared with unboiled). Whatman 0.45 μm pore size cellulose nitrate filters were used in preference to the comparable filters from Millipore for economic reasons.

The [^{3}H]choline uptake assays were routinely performed at 30^{o}C as previously described (Breer, 1982b), though 37^{o}C and 21^{o}C have been used with insect synaptosomes for [^{3}H]GABA (Gordon et al., 1982) and [^{3}H]choline uptake studies (Dwivedy, 1985) respectively. The uptake of [^{3}H]choline by the homogenate was studied over a range of incubation temperatures, with the aim of defining the optimum temperature, as the uptake of [^{3}H]choline by rat hippocampal synaptosomes increases as the incubation temperature is increased from 17^{o}C to 37^{o}C (Simon & Kuhar, 1976). However, above 30^{o}C the uptake increased with increasing incubation temperature, up to 50^{o}C, before being abolished at 70^{o}C (Fig.38). What the fate of the [^{3}H]choline is, once inside the synaptosomes at 50^{o}C, is unknown, but the elevated uptake at 50^{o}C compared with the uptake at 30^{o}C was blocked by HC-3. Therefore it appears that the effect is dependent on the [^{3}H]choline transporter. One should not forget that the homogenate is derived from the desert locust which is an ectotherm which in its natural habitat must endure temperatures in excess of 40^{o}C. This

phenomenon requires more work before the results can be used practically. While, therefore, subsequent uptake experiments were carried out at 30°C, we should perhaps begin to consider what the 'standard' conditions are when working with insects.

The uptake processes of mammalian brain are generally susceptible to metabolic perturbation as they require energy to drive them. However, no consistent effects on [^{3}H]choline uptake were observed when glucose was omitted from the incubation buffer, sodium azide was added or the buffer was gassed with O_2/CO_2 (Table 13). This is in agreement with the results of Breer (1982b) who concluded that insect synaptosomes have an unusually high metabolic resistance, in contrast to mammalian synaptosomal [^{3}H]choline uptake which is inhibited by glucose removal (Yamamura & Snyder, 1973).

3.4.5/ The release of radioactivity from [^{3}H]choline-loaded homogenate

If [^{3}H]choline is being taken up by a high affinity transport system into synaptosomes in the homogenate, then by analogy with the mammalian system, one would expect this [^{3}H]choline to be converted into [^{3}H]acetylcholine (ACh). Furthermore the synaptosomes should release this [^{3}H]ACh on stimulation with elevated K^+ (Mulder et al., 1974; Wonnacott & Marchbanks, 1976).

When the homogenate was preloaded with [^{3}H]choline, the radioactivity released in the presence of elevated K^+ was significantly greater than that released in the standard uptake buffer. The radioactivity was collected and the choline species were extracted and separated by tlc.

The extraction of choline esters with tetraphenylboron in heptanone, followed by re-extraction with HCl, is greatly reduced by K^+ (this problem can be alleviated by using allylcyanide as a solvent, Marchbanks & Israel, 1971). Furthermore the amount of acetylcholine extracted is reduced by the presence of NaCl in the sample (Fonnum, 1969). Though precise comparisons with the work of Fonnum (1969) cannot be made under the experimental conditions reported here, it is expected that there would be 60% extraction from the Na^+-stimulated sample and ~40% from the K^+-stimulated sample (assuming that all the radioactivity is in the form of choline). However, the affinity of tetraphenylboron for choline esters increases with increasing size of the acyl group (Fonnum, 1969). Therefore if the K^+-stimulated sample possesses a greater proportion of ACh than the Na^+-stimulated sample, this would compensate for the greater interference of K^+ than NaCl with the extraction. It also explains the low extraction obtained with the standard which was [^{3}H]choline. The additional extractions which were necessary to get a sufficiently

clean sample for tlc resulted in low (1-8%) net recovery, from the tlc plate. The various differences in the extraction procedure preclude quantification of the release by this extraction and tlc method; neither can a comparison be made of the absolute amounts of different choline esters obtained from different buffers. That is, the quantities of ACh extracted from the Na^+- and K^+-stimulated samples cannot be directly compared. On tlc, radioactivity was always obtained in the ACh spot, suggesting that the [^{3}H]choline taken up by the homogenate is converted to ACh, which is a feature of the high-affinity choline uptake system of mammalian brain (Meyer et al., 1982). Furthermore the ratio of the radioactivity recovered from the ACh spot to that obtained from the choline spot was always greater in the K^+-stimulated sample than in the Na^+-stimulated sample. This is very similar to the results of Mulder et al. (1974) with rat hippocampal synaptosomes, suggesting that elevated K^+ stimulates ACh release from the locust preparation. The ACh obtained in the Na^+-stimulated sample represents the background leakage from synaptosomes due to their being damaged during the release manipulations.

It is concluded that the synaptosome-rich homogenate is capable of [^{3}H]choline uptake, and that the [^{3}H]choline taken up can be released, partly in the form of [^{3}H]ACh, by elevated K^+, as has been demonstrated previously for insect synaptosomes (Breer &

Knipper, 1984). This demonstrates that the preparation is physiologically viable and that the observed [^{3}H]choline uptake is a valid screen for physiological viability.

3.4.6/ The uptake of [^{3}H]GABA by the homogenate

Once the homogenate, H, had been shown to be physiologically viable by [^{3}H]choline uptake and the K^+-stimulated release of [^{3}H]ACh, it was possible to make a preliminary study of [^{3}H]GABA uptake by the homogenate.

When incubated with 0.1 μM [^{3}H]GABA at 30°C, the homogenate accumulated 8 pmol [^{3}H]GABA/mg protein over a 20 min time course (Fig.40). This uptake was greatly reduced by incubation on ice (as one would expect of an active transport process) and by elevated (50 mM) K^+, which should cause the synaptosomes to be depolarized. The time course of [^{3}H]GABA uptake by the homogenate is similar to that observed in the early studies on mammalian brain preparations. The uptake of [^{3}H]GABA by brain slices at 25°C saturates over a 60 min time course (Iversen & Neal, 1968; Bond, 1973). In synaptosome preparations the uptake sites might be expected to be more readily accessible to the ligand than in brain slices. Rat brain synaptosomal [^{3}H]GABA uptake, at 27°C, saturates over a 20 min time course (Martin & Smith, 1972) while nerve ending particles from cat brain exhibit a linear acumulation of [^{3}H]GABA at

37°C over a 20 min time course (Snodgrass et al., 1973). Such time courses of uptake, however, are in contrast with the two previous reports of [^{3}H]GABA uptake by insect synaptosomal preparations (Gordon et al., 1982; Breer & Heilgenberg, 1985) in which the uptake was saturated after 3 min and 2 min incubation periods respectively. The study of Gordon et al. (1982) was conducted at 37°C, which might explain a more rapid uptake than that reported here, though Breer & Heilgenberg (1985) used a 30°C incubation temperature. The study of Gordon et al. (1982) did not utilize a GABA-T inhibitor to prevent any free endogenous enzyme in the preparation depleting the [^{3}H]GABA. The absence of such an inhibitor, however, did not make any major difference to the [^{3}H]GABA uptake by the homogenate (Fig.41) which is a cruder preparation than that of Gordon et al. (1982) and would therefore be expected to contain more free GABA-T.

The uptake of [^{3}H]GABA by locust synaptosomes exhibits a peak after 5 min incubation time (Whitton, personal communication) and the subsequent decrease in the uptake was attributed to the presence of free endogenous GABA-T. Recently though, Zhu et al. (1986) reported that the high affinity uptake of [^{3}H]GABA by mouse brain synaptosomes was inhibited by preincubation for 15 min with 10 μM aminooxyacetic acid (AOAA). In contrast, Loscher (1980) demonstrated that while the GABA-T inhibitor, gabaculline, inhibited [^{3}H]GABA uptake

in a crude mitochondrial fraction from rat brain with an IC_{50} of 81 μM, AOAA had no effect. When [^{3}H]GABA uptake was studied in the locust homogenate in the presence of 50 μM AOAA, 10 μM gabaculline and with no GABA-T inhibitor present, there was no significant difference in the uptake under any of the conditions. Snodgrass & Iversen (1973) demonstrated that AOAA did affect the uptake of [^{3}H]GABA by rat brain slices, but only at high concentrations (optimal at 2 mM) and following incubation of the slices for at least 15 min prior to the addition of [^{3}H]GABA. The authors postulated that this effect on uptake was a long-term secondary effect via an action of AOAA on pyridoxal phosphate. The locust homogenate was not preincubated with the GABA-T inhibitor. Indeed, it was hoped that preincubation without the inhibitor might result in a reduction of the endogenous GABA by the action of any GABA-T in the preparation. It is concluded that, under the uptake conditions utilized in this study, there is no significant effect of GABA-T inhibitors or of GABA-T on the observed uptake.

Nipecotic acid is an inhibitor of both glial and neuronal [^{3}H]GABA uptake in mammalian brain preparations (Larsson et al., 1985) whereas 2,4-diaminobutyric acid (DABA) exhibits 10-fold greater affinity for neuronal than for glial uptake (Schon & Kelly, 1974). Glial uptake of [^{3}H]GABA was thought to be specifically inhibited by β-alanine (Enna & Snyder,

1975) but more recently this has been disproved by use of neuronal and astrocytic cultures (Griffiths et al., 1986). Therefore there was no specific glial uptake inhibitor available to test on the [^{3}H]GABA uptake in locust homogenate (though recently 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol has been shown to be a specitic glial uptake inhibitor, Schousboe et al., 1986). DABA inhibited the uptake of [^{3}H]GABA by the locust homogenate to almost the same extent as incubation on ice (Fig.42), supporting the concept that the observed uptake is by a high attinity neuronal transport system. The action of nipecotic acid at the same high concentration as DABA (0.1 mM) was less clear cut. Nipecotic acid had little effect on the initial [^{3}H]GABA uptake but may have some effect on a later component of the uptake. As nipecotic acid inhibits both neuronal and glial uptake in mammalian brain, it is difficult to rationalize the action of nipecotic acid on the locust uptake unless the insect neuronal GABA uptake system ditters from its mammalian counterpart in being insensitive to nipecotic acid.

There is always the possibility that any subfraction of central nervous tissue possesses fragments of glial membrane which have sealed to form 'gliasomes' (Jones, 1975; Morgan, 1976) which contribute to the observed uptake of [^{3}H]GABA. The only method of truly distinguishing between glial and neuronal GABA uptake is to use glial and neuronal cell cultures

(Larsson et al., 1985). The locust homogenate probably contains glial elements and the only evidence against any such elements contributing to the observed [^{3}H]GABA uptake is that it is virtually abolished by DABA. The preparation will also contain free mitochondria which are known to accumulate GABA. However, Levi et al. (1974) demonstrated that synaptosomes exhibit a 10-fold greater uptake activity than mitochondria in various regions of rat brain. Kuriyama et al. (1969) reported that synaptosomes only take up 2.5-fold greater amounts of GABA than mitochondria but, to demonstrate this, they had to use 700 x greater concentrations of [^{3}H]GABA than used in the locust homogenate. Mitochondria from rat cerebral tissues have been reported to be 1 μm by 2.5-4 μm (McIlwain & Bachelard, 1971). Though structures of such size may be destroyed during the homogenization, there remains the possibility that mitochondria contribute to the observed [^{3}H]GABA uptake, as they may be retained on a 0.45 μm pore size filter. Throughout the time courses of [^{3}H]GABA uptake, it became apparent that there was a consistent sigmoidicity in the curves and hence the graphs were drawn to illustrate this (Figs.40-42). Heterogeneity in the affinities of the uptake systems is unlikely to be the cause of this as such heterogeneity would only be apparent when varying the concentration of [^{3}H]GABA rather than the time. If the sigmoidicity is real, the most likely explanation is that there is a heterogeneity in the structures in the preparation.

Thus, it there were two different sizes of membrane enclosed compartment one would not observe a linear accumulation of ligand. The smaller compartment should fill first, however, such that the curve would look different from that observed here. If, on the other hand, membrane enclosed structures were forming during the incubation one would expect to see an increase in the uptake with respect to time as observed here. This could be tested by allowing a longer preincubation time which should abolish the sigmoidicity.

It is concluded that the locust ganglionic homogenate possesses a [^{3}H]GABA uptake capacity which is blocked by the mammalian neuronal uptake inhibitor, DABA. By analogy with mammalian synaptosomes, it is expected that [^{3}H]GABA would be released from the preparation by elevated K^+, in a partially Ca^{2+}-dependent manner. When a preliminary study was made of this, the use of a centrifugation method caused too greater disruption of the synaptosomes, resulting in very high background 'release'. This was partially alleviated by the use of a gentler filtration technique (Table 16). Although the released radioactivity was always greater in the presence of elevated K^+ when Ca^{2+} was present than when it was absent, and the release was always greater in elevated K^+ than in standard buffer, the differences were small and more work is required on this system.

3.4.7/ Future studies with insect synaptosomes

The experiments presented in this thesis represent a very preliminary study of insect synaptosomes. However, using [^{3}H]choline uptake as a screen for the physiological viability of the synaptosomes, the optimal physical parameters for this viability can be more fully elucidated. The optimal osmotic strength of the buffer could be ascertained for example.

A homogenate is now available which is known to be rich in synaptosomal profiles. The kinetic and pharmacological characteristics of the [^{3}H]GABA uptake capacity of this preparation should be elucidated more fully. The homogenate, however, is a very heterogeneous preparation which makes the interpretation of the results obtained with it difficult. Therefore, it may be preferable to now proceed with the purification of the synaptosomes from the homogenate. The best strategy to achieve this may be to limit the number of manipulative steps and apply the homogenate directly onto a Ficoll gradient, and effectively go from the homogenate directly to the final stage of the preparative procedure of Breer & Jeserich (1980).

Fractionation of insect nervous tissue will always carry the risk of producing heterogeneous preparations which contain both glial and neuronal elements. At present we have no knowledge of the relative contribution that neurones and glia make to the

uptake of GABA in insect CNS. Cultures of insect central neurones have already been developed (Beadle & Lees, 1986). When glial cell cultures are developed from insect brain then a parallel study might be made on glial and neuronal GABA uptake, as has been performed with cultures from mammalian CNS (Larsson et al., 1985).

An ultimate aim in developing stable synaptosomes from insect CNS must be to have the capacity to continuously perfuse them, as is routinely done with mammalian brain synaptosomes (Rateiri et al., 1974; Redburn et al., 1975; Minnema & Michaelson, 1985; Rapier et al., 1985). This is an extremely powerful technique, permitting the analysis of the mechanisms of presynaptic control of the release of the radiolabelled transmitter with which the synaptosomes are pre-loaded. A crude superfusion of insect synaptosomes, preloaded with [^{3}H]choline, on swollen Sephadex G-15 has already been published (Breer & Knipper, 1984). The lack of stability of current insect synaptosome preparations has precluded the application of the advanced mammalian synaptosome technology to the insect preparations, but the arrival of such technology should herald a new era in insecticide development.

CHAPTER 4

OVERVIEW - APPLICATIONS AND CONCLUSIONS

In the opening chapter to this thesis, a brief background was given to the pesticide industry and a case was made for the need for basic research into hitherto uncharacterized invertebrate neurotransmitter systems; GABA is one such system. In support of this, insect GABA receptors are already demonstrating applications in the elucidation of the mode of action of pesticides.

4.1/ The action of pesticides

The chlorinated hydrocarbons are a major class of insecticides whose actions may involve perturbation of GABAergic transmission. Cyclodienes (such as endrin) and γ-hexachlorocyclohexane (lindane) have been shown to affect mammalian GABA receptors by potently displacing [^{3}H]dihydropicrotoxinin ([^{3}H]DPTXN) binding (Matsumura & Ghiasuddin, 1983) and [^{35}S]TBPS binding in rat brain membranes (Abalis et al., 1985b). With the availability of comparable binding assays in invertebrate nervous tissues it is now possible to compare the effects of these compounds in binding and physiological studies on the same tissues. Preliminary work in this direction has shown some chlorinated hydrocarbons to be active in displacing [^{3}H]DPTXN and [^{35}S]TBPS binding from insect membranes, though the relative potencies are different from those reported in analogous vertebrate studies (Tanaka et al., 1984; Cohen & Casida, 1986; Szamraj et

al., 1986). Hopefully further work utilising the invertebrate techniques now available will lead to the elucidation of the mode of action of these insecticides.

A second class of pesticides which has received a lot of attention recently is the avermectins (AVMs). These macrocyclic lactones are extremely potent against a variety of parasitic nematodes and arthropods. Electrophysiological evidence for their action at GABA receptor chloride channels came from work on lobster (Fritz et al., 1979) and nematodes (Kass et al., 1980). However initial biochemical evidence for a GABAergic mode of action was obtained in mammalian preparations. AVMs were shown to increase the release of GABA from rat brain synaptosomes (Pong et al., 1980), enhance the in vivo muscle relaxant effects of benzodiazepines in mice (Williams & Yarborough, 1979), modulate the binding of TBPS and GABA (Pong & Wang, 1982; Drexler & Sieghart, 1984ab; Calcott & Fatig, 1984; Olsen & Snowman, 1985) and enhance benzodiazepine binding in dog and rat brain preparations (Williams & Yarborough, 1979; Paul et al., 1980; Pong et al., 1981, 1982; Supavilai & Karobath, 1981; Williams & Risley, 1982, 1984; Drexler & Sieghart, 1984c). Comparable data in invertebrate preparations are limited though. AVMs have been shown to potently inhibit honeybee brain [^{3}H]muscimol binding (IC_{50} 3nM, Abalis & Eldefrawi, 1986), while at 0.1 - 5μM they show enhancement of [^{3}H]GABA binding in cockroach CNS (Lummis & Sattelle, 1985b). No effect on housefly or crayfish

[^{35}S]TBPS binding was observed by Szamraj et al. (1986), nor was there any modulation by AVM of [^{3}H]muscimol binding in these tissues (Olsen, personal communication). No effect of AVM was found in cockroach ganglia on the binding of [^{3}H]DPTXN, [^{3}H]muscimol or [^{3}H]diazepam (Tanaka & Matsumura, 1985). [^{3}H]Ivermectin (5nM) exhibits specific binding to locust ganglionic membranes while the unlabelled ligand enhances [^{3}H]FNZP binding in the same tissue (Scott, Robinson & Lunt, unpublished observation). However, in a recent review of the mode of action of the AVMs, Wright (1986) concluded from the electrophysiological evidence that AVMs had two distinct actions in vertebrates and invertebrates. These two postulated actions include a reversible effect at concentrations in the 10nM range which appears specific to GABA receptors, whereas an irreversible effect is suggested to occur at higher concentrations. This latter effect might involve glycine (inhibitory) responses in vertebrates and glutamatergic (excitatory) responses in invertebrates. This conclusion is consistent with the more recent data of Beadle & Lees (1986) obtained from locust neuronal cultures in which AVM acts as a GABA-mimetic but it may act on two separate populations of channels. Also its action is partially an irreversible one on a population of channels that does not respond to GABA. Furthermore, although Mellin et al. (1983) demonstrated that AVM opened a picrotoxin-sensitive chloride ion channel in lobster

muscle, Chalmers et al. (1986) showed that AVM potentiated GABA responses in crayfish muscle but not in stretch receptor neurones, despite being able to increase chloride conductance on its own in both tissues. Tanaka & Matsumura (1985) demonstrated in cockroach leg muscles that AVM increased chloride permeability, apparently independently of GABA receptors, and Duce & Scott (1985) showed that AVM activated chloride channels in a variety of insect muscles including some that lacked inhibitory GABAergic innervation. Abalis et al. (1986) have reported that in rat brain, AVMs do open a chloride channel by binding to a GABA receptor and acting as a partial agonist, but that they also open voltage-dependent chloride channels which are totally insensitive to GABA. In conclusion, it would appear that the mode of action of the avermectins is only partially related to the GABA receptor complex.

As our biochemical knowledge of invertebrate GABAergic transmission increases, so our practical ability to measure its various parameters increases. Hopefully this will not only help us in our understanding of the mode of action of existing pesticides, as outlined above, but perhaps more importantly might open new doors for the rational design of novel, specific pesticides.

4.2/ Conclusions on insect central GABAergic neurotransmission

In 1973 Gerschenfeld concluded from the then available, largely electrophysiological, data that GABA was probably the universal inhibitory transmitter of the invertebrate neuromuscular junction, excluding molluscs, and that the evidence was particularly convincing in the arthropods. The situation in invertebrate central neurotransmission was less clear; GABA was probably involved in crustacean and insect CNS whereas its role in molluscan CNS was doubtful. Certainly there was scant knowledge of any of the biochemical components of any invertebrate GABA system (Gerschenfeld, 1973).

The work described in this thesis has been concerned with insect central GABA receptors and the means of studying insect central GABA uptake and release mechanisms. The future potential and the limitations of the work have already been discussed in the relevant chapters. In addition, however, the work is part of the recent growth of interest in this field. Our knowledge of GABAergic transmission in insect CNS has come a long way since 1973 and we are beginning to see a picture of a GABAergic system in insect CNS which in overall organization is similar to that outlined in Fig.1 for the mammalian nervous system.

We now know that GABA is present in significant quantities in insect CNS. In the last year

we have obtained evidence that insect brain possesses the synthetic and metabolic machinery comparable with that involved in mammalian brain GABAergic neurotransmission (see Chapter 1). Though insect GAD and GABA-T have overall similarities with the mammalian central enzymes, subtle differences are emerging in the fine tuning of the enzymes, not only between vertebrate and invertebrate CNS, but also between different invertebrate classes. We are starting to see the mapping of invertebrate GABAergic neurones both with antibodies to GAD (Stapleton, 1986) and monoclonal antibodies to GABA (Meyer et al., 1986). In the future these approaches should provide a more precise picture of the role of GABA in invertebrate nervous tissues.

The work in this thesis has contributed to the growing case for an insect central GABA receptor complex. Here again, as in any comparisons between the emerging insect GABA system and its established mammalian counterpart one sees an overall similarity but with subtle differences. Such differences in GABAergic enzymes are being characterized, but the insect uptake systems and receptors are much harder to study. In the future insect synaptosomal preparations of greater stability than are presently available might permit not only the full characterization of GABA uptake but also investigation of the mechanisms of insect neuronal GABA release. Greater exploitation of invertebrate neuronal and glial cultures would not only allow the

pharmacological characterization of GABA receptors but also provide an alternative vehicle for uptake studies.

In the future then we will hopefully see the full characterization of invertebrate GABAergic neurotransmission. This is not only of applied importance in the development of pesticides, but also essential from a comparative standpoint in our understanding of the evolution of such transmission systems.

APPENDIX 1

GABA RECEPTORS ON LOCUST MUSCLE

A.1/ INTRODUCTION

In addition to the central actions of GABA in the insect nervous system, we have known for some time that GABA is the major inhibitory neurotransmitter at the insect neuromuscular junction (Usherwood & Grundfest, 1964, 1965). Indeed for many years our knowledge of the peripheral GABA receptors far outweighed any knowledge of the corresponding central receptors because the insect neuromuscular junction is much more accessible to the electrophysiologist than the brain (see Gerschenfeld, 1973).

The first biochemical study of putative GABA receptors in insects were carried out in muscle preparations (De Robertis & Fizer de Plazas, 1974; Abalis et al., 1983). Abalis et al. (1983) described the binding of [H]flunitrazepam ([^{3}H]FNZP) to a housefly thoracic mebrane preparation. Although the binding was enhanced by GABA, no binding of [^{3}H]GABA or [^{3}H]muscimol was observed in the same preparation. Therefore, in addition to the studies of insect central GABA receptors described in Chapter 2 of this thesis, a study was made of GABA receptors in locust muscle. The binding of [^{3}H]muscimol and [^{3}H]FNZP were investigated in membranes prepared from locust flight muscle, as this would be most directly analogous to the tissue preparation of Abalis et al., (1983). Such a study might permit a comparison between the putative GABA receptors in locust

CNS (this thesis) and in housefly flight muscle (Abalis et al., 1983).

It has not been established whether or not housefly flight muscle has any inhibitory nervous input, but GABA is known to be the inhibitory neurotransmitter in the extensor tibiae (jumping) muscle (ET) of locust leg (Usherwood & Grundfest, 1965). Therefore an electrophysiological study was made of GABAergic responses in locust ET, to gain information on the pharmacology of known insect muscle GABA receptors. Such pharmacology might then be compared with the pharmacologies of any GABA binding sites detected in binding studies on locust flight muscle.

A.2/ BINDING STUDIES ON FLIGHT MUSCLE MEMBRANES

A.2.1/ Preparation of the membrane fraction, P_2M, from locust flight muscle

The head, abdomen and legs of the locust were cut off and the thorax was cut ventrally and pulled open to reveal the muscles. Most of the fat body was removed by blotting with tissue papers and the entire thoracic muscle dissected out and stored on ice. The muscle (approximately 3 g from 20 locusts) was homogenized (3% w/v) in locust saline (Table 17) in a Potter-Elvejham homogenizer with a motor driven pestle (7 x 10 strokes, speed, 500 rpm; radial clearance, 0.15 mm). The homogenate was then filtered through nylon bolting cloth (192 μm mesh) and the filtrate subjected to the fractionation scheme shown in Fig.43.

0.15M sodium chloride
10mM potassium chloride
2mM calcium chloride
5mM magnesium chloride
4mM potassium hydrogen carbonate
6mM potassium dihydrogen phosphate
pH7.0, adjusted with 1 M NaOH.

Table 17 Composition of locust saline, after Hoyle (1953).

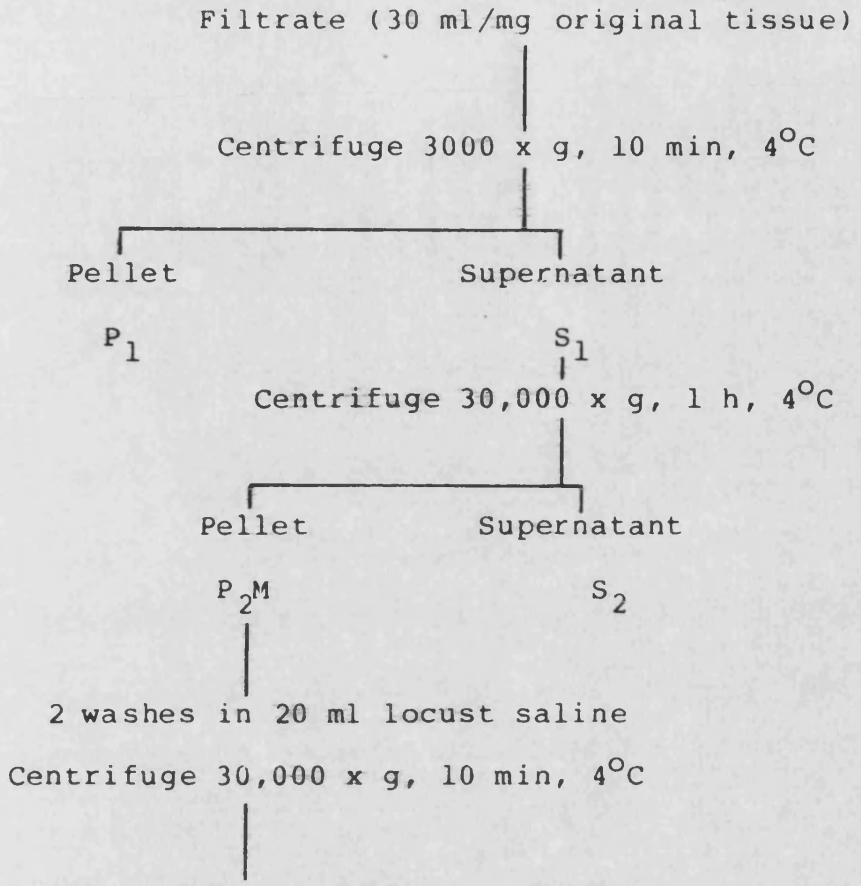


Fig.43 Fractionation scheme for locust muscle, to prepare the membrane fraction, P_2M.

A.2.2/ The binding of [^{3}H]muscimol and [^{3}H]FNZP to P_2M

The binding of [^{3}H]muscimol to the membrane fraction, P_2M, from locust flight muscle was assayed as described in 2.2.3, using a 400µl sample of the resuspended membranes (see above). Total and non-specific binding are compared in Fig.44. The apparent specific binding was 5% of the total binding. If such specific binding is real, it is low level, non-saturating binding over the 1-20 nM [^{3}H]muscimol concentration range.

The binding of [^{3}H]FNZP to P_2M was assayed as described in 2.2.4., using Tris-HCl buffer containing 1 mM EGTA rather than 4 mM $CaCl_2$ and 4 mM $MgCl_2$. Indeed when EGTA was replaced with $CaCl_2$ and $MgCl_2$ (as proved necessary to obtain specific binding of [^{3}H]FNZP to ganglionic membranes) the binding of [^{3}H]FNZP to P_2M became more variable. The effects of diazepam, Ro5-4864 and clonazepam on the binding of [^{3}H]FNZP to P_2M are shown in Table 18.

COMPOUND	IC_{50}(µM)
Diazepam	0.01
Ro5-4864	0.23
Clonazepam	>1.0

Table 18 The concentrations of diazepam, Ro5-4864 and clonazepam giving 50% displacement (IC_{50}) of the binding of 10 nM [^{3}H]FNZP to P_2M. Results are the means of two experiments employing triplicate assays.

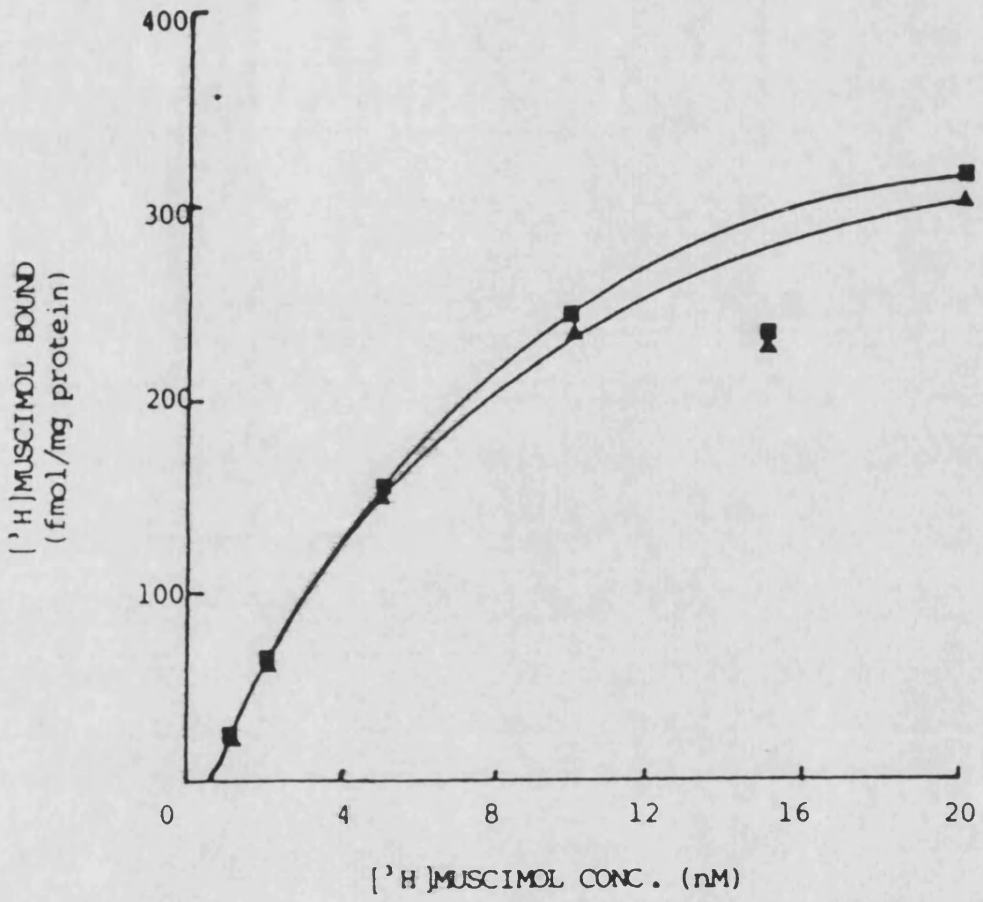


Fig.44 The total (■), and non-specific (▲) binding of 1-20 nM [^{3}H]muscimol to P_2M. Results are the means of 2 experiments. Assays were performed in triplicate.

A.2.3/ Determination of the tissue concentrations of calcium and magnesium

The concentrations of calcium and magnesium in locust flight muscle and the membrane preparation, P_2^M derived from it, were measured by atomic absorption spectroscopy as described in 2.2.5. The resulting histograms are shown in Fig.45.

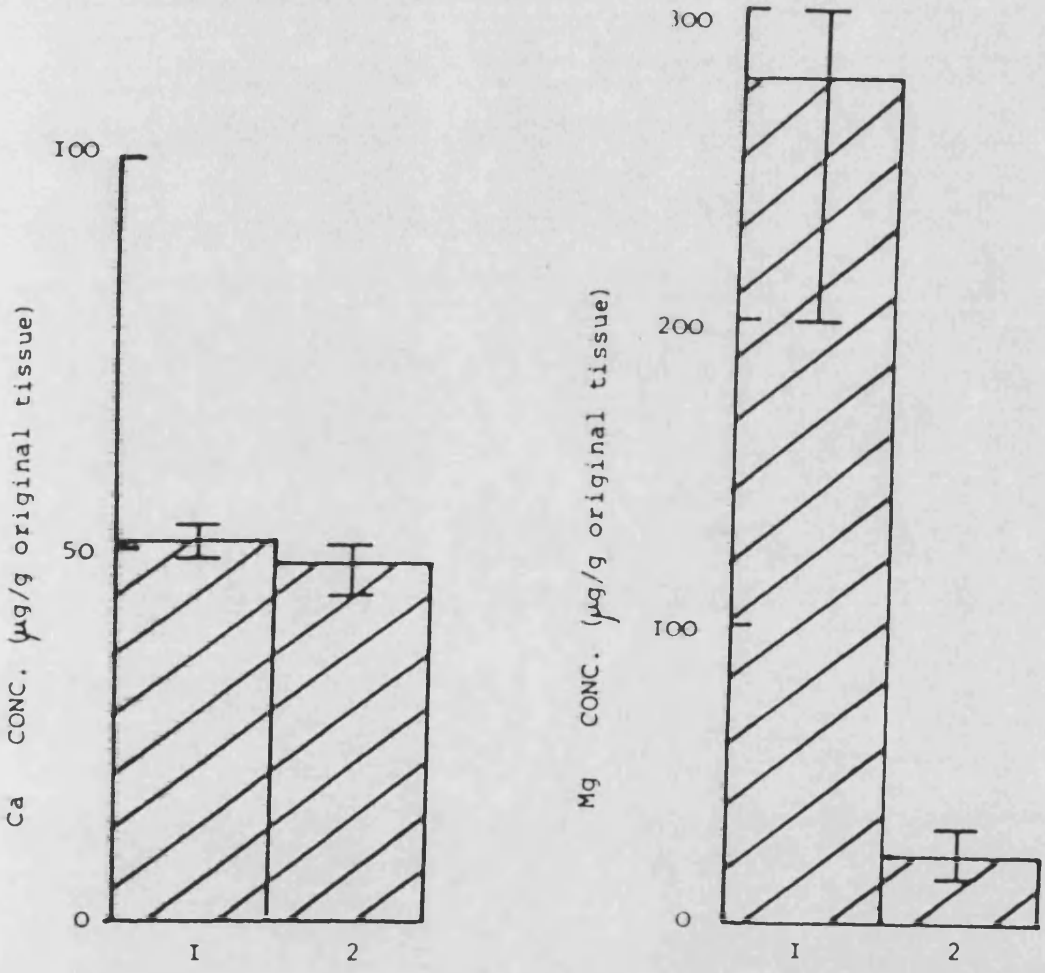


Fig.45 Concentrations of calcium and magnesium in 1) locust flight muscle and 2) P_2M. The histogram shows the mean result of three separate determinations. Bars denote the upper and lower values of the three results.

A.3/ ELECTROPHYSIOLOGICAL STUDIES ON THE EXTENSOR TIBIAE MUSCLE

A.3.1/ The organization of the muscles and nerves of the locust femur

The distributions of the nerve and muscle fibre types in locust jumping muscle have been extensively characterized (see Hoyle, 1978). Briefly the femur of the locust contains three muscles. Removal of the flexor tibiae and retractor unguis muscles reveals the third muscle, the extensor tibiae (ET, the jumping muscle) (Fig.46a). Four axons innervate the ET: fast (FETi), slow (SETi), common inhibitor (CI) and the dorsal unpaired median (DUMETi) (Fig.46b).In studying GABAergic responses one is interested in those muscles which receive inhibitory innervation and therefore in the case of the ET, those muscle fibres which are innervated by the CI axon. The ET is composed of discrete muscle bundles and, amongst others, bundles 32, 33, and 34 have a defined inhibitory input (Duce & Scott, 1985) as do the small distal muscle units 135c&d. Muscle bundle 34 was used in this study, unless stated otherwise.

A.3.2/ The extensor tibiae preparation

The metathoracic leg was cut from the locust at the joint between the coxa and the body and pinned out on sylgard in a disposable perfusion bath. The ET

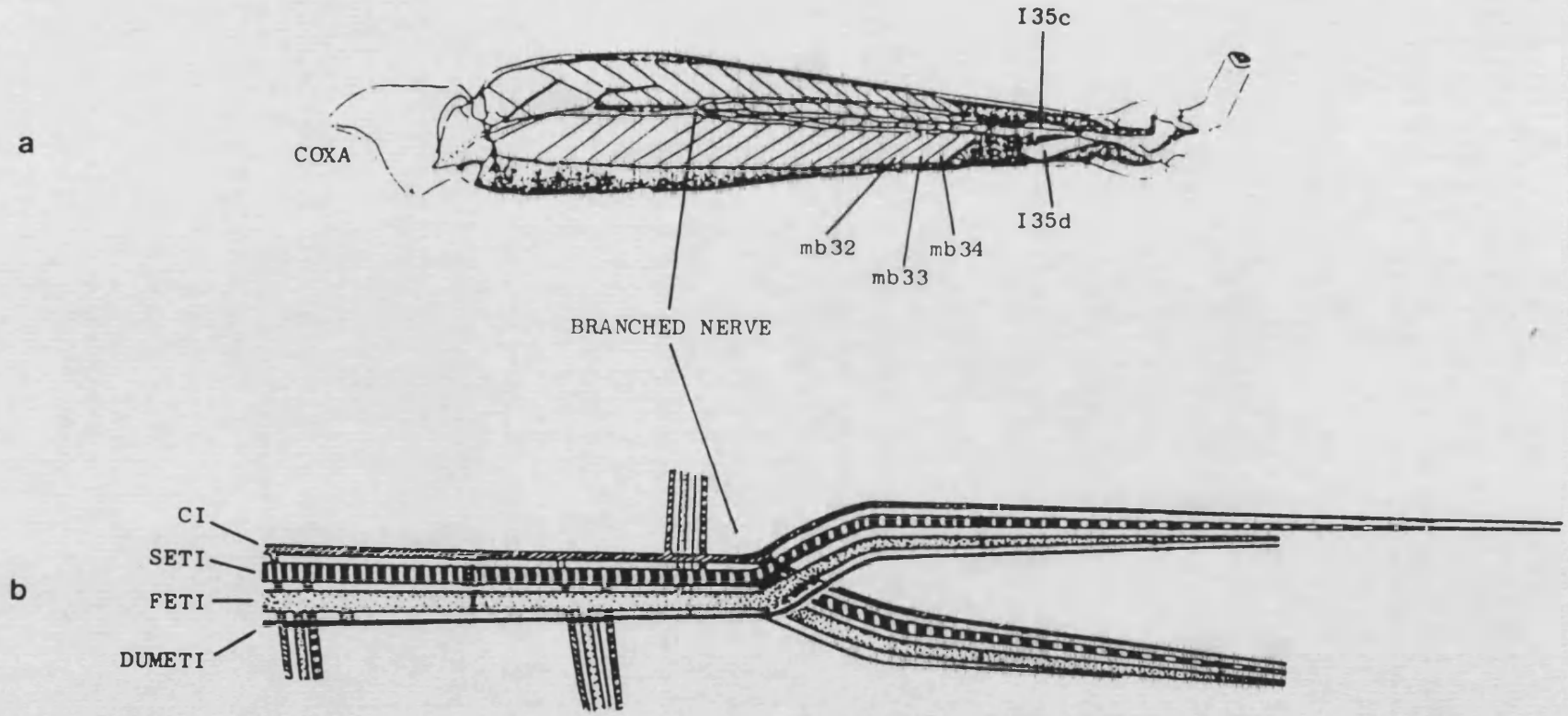


Fig.46 a) Diagram of the dissected locust femur preparation, showing the exposed muscle bundles of the ET muscle. Muscle bundles 32, 33, 34 and I35c&d are indicated.

b) Diagram of the different axons which innervate the ET, corresponding to the branched nerve visible in the dissected femur (a).

was exposed by removing the cuticle and dissecting away the overlying flexor muscles and main trachea to give the preparation shown in Fig.46a. The muscle bundles were then extended by pinning out the cut edges of the cuticle and the preparation perfused with saline (170mM NaCl, 10mM KCl, 2mM $CaCl_2$, 10mM $MgCl_2$, 10mM HEPES, adjusted to pH6.8 with 1M NaOH, after Duce & Scott, 1985).

A.3.3/ Intracellular recordings

Intracellular recordings were made from muscle bundles 32, 33, 34 and 135c&d using a glass microelectrode filled with filtered 3M KCl. This electrode was connected to an oscilloscope via a voltage follower and the circuit completed with a Ag/AgCl indifferent electrode immersed in the perfusion bath.

This simple system was used for an initial study with the intention of observing miniature inhibitory post-synaptic potentials (mipps), caused by the passive endogenous release of discrete quanta of GABA. Glutamate will also be released causing miniature excitatory post-synaptic potentials and so the preparation was perfused with saline containing 1mM glutamate to desensitize the glutamate receptors.

Despite making intracellular recordings from muscle bundles 32, 33, 34 and 135c&d, no mipps were observed in any preparation. Therefore it was decided to measure neurally evoked inhibitory post-synaptic

potentials (IPSPs).

A.3.4/ Neurally evoked IPSPs

Because mipps could not be detected in the ET preparation (see above) an attempt was made to study neurally evoked IPSPs whereby a nerve containing the CI is cut and stimulated with an electric current; one should observe a response in any cell which is innervated by that nerve.

The preparation was perfused with saline containing 1mM glutamate as described above to prevent contraction of the muscle on stimulation of the nerve. The common inhibitor axon was cut and stimulated with a 30 nA current pulse, whilst making intracellular recordings as before (Fig.47). Despite making recordings from several different muscle bundles and cutting the nerve both in the coxa, where it fuses with the axons of the other nerves, and at several different points along its length in the femur, no IPSPs were observed.

It was thought that this lack of success might be due to the HEPES buffer acting as a GABA agonist because it is an aminosulphonic acid and might mask any GABAergic responses. Therefore HEPES was replaced by 10 mM Tris in the perfusion saline but still no IPSPs were observed. A further possibility was that the high glutamate concentration was connected in some way with the failure to detect IPSPs. Lack of time prevented any further study of this and

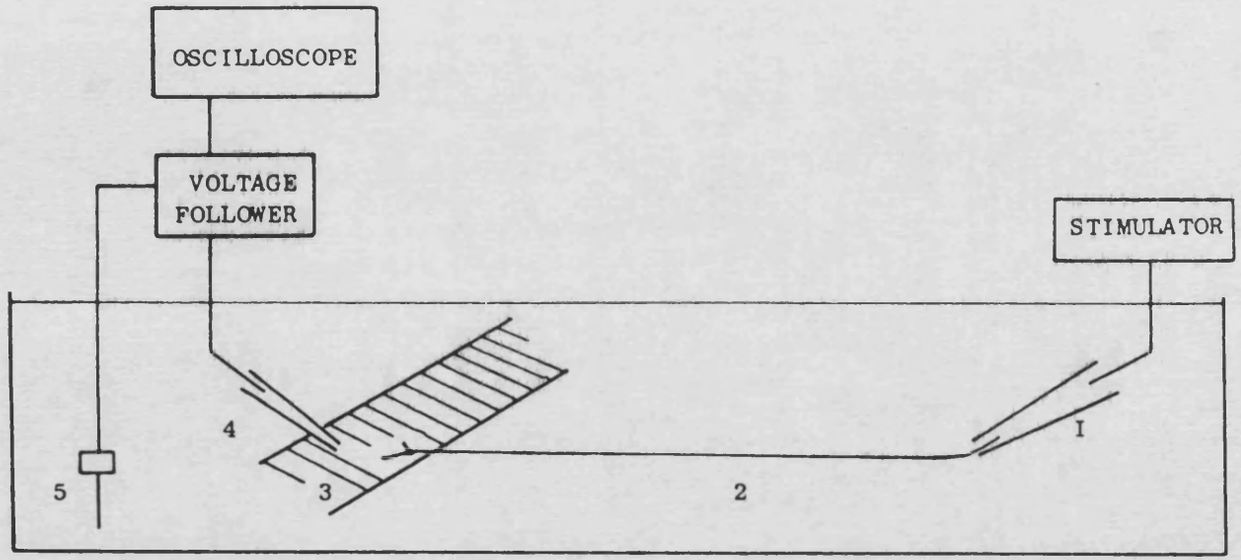


Fig.47 Diagram of the system for measuring neurally evoked IPSPs in muscle bundle 34 of the locust extensor tibiae.
I - Glass suction/stimulation electrode (containing a $Ag/AgCl_2$ wire)
2 - Common inhibitor axon
3 - Muscle bundle 34
4 - Glass recording electrode (3 M NaCl)
5 - $Ag/AgCl_2$ indifferent electrode

therefore the glutamate was removed from the saline and the effects of bath-applied GABA were studied.

A.3.5/ Bath application of GABA

Intracellular recordings were made from muscle bundle 34 while perfusing with saline containing GABA. A hyperpolarization was observed on application of GABA at concentrations $\geq 1\mu M$; for example the membrane potential fell from 48mV to 58mV on applying 1 μM GABA and then returned to 48mV after removal of the GABA on washing. However no hyperpolarization was observed on a second application of GABA to the same preparation. This was a consistent effect despite extensive washing or using sequentially higher concentrations of GABA. It is known that locust muscle GABA receptors are not desensitized by exposure to GABA (Usherwood, 1975). The lack of hyperpolarization of the muscle on a second application of GABA may possibly be due to the membrane potential in these muscle cells being very close to the equilibrium potential for chloride ions (E_{Cl}). Therefore on application of GABA, GABA-gated chloride channels open and the cell hyperpolarizes due to the influx of chloride ions into the cell. The chloride ion concentration, however, has now equilibrated. Therefore, on a second application of GABA, though the chloride channels open, there is no observed change in membrane potential. However there should still be an accompanying change in the resistance of the membrane and it is

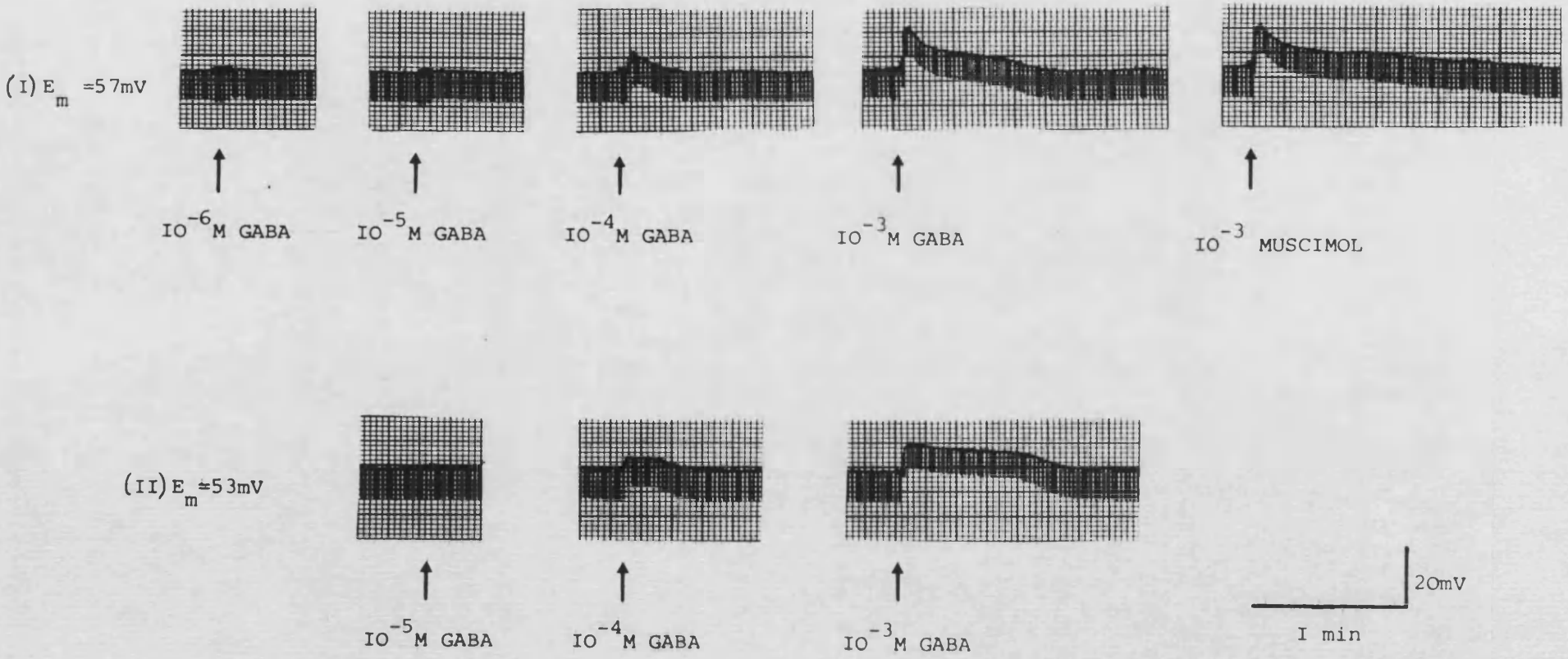
possible to measure this in addition to the membrane potential.

A.3.6/ Resistance changes caused by constant current injection

A single recording electrode was used to record from muscle bundle 34 and constant current pulses of 30nA were injected into the cell with the same electrode. The resulting resistance changes were recorded and the effects of GABA and other drugs on these resistance changes were investigated.

Initially the recording electrode contained 3 M KCl and GABAergic responses were observed in this preparation (Figs.48-50). Some cells responded to GABA at 1 μM while others only responded to 0.1 mM GABA (Fig.48). Muscimol acted as a GABA mimetic though the effects of muscimol took longer to wash off than those of GABA (Figs.48&49). Picrotoxin (1 mM, Fig.49) and diazepam (0.1 mM, Fig.50) had no significant effects on the responses of the cells studied to 1 mM GABA. Bicuculline (0.1 mM) caused a reduction in the change in resistance and delayed the response to 0.1 mM GABA (Fig.50). Throughout the work using a KCl recording electrode a consistent feature of the observed GABA reponses was that while GABA caused a change in membrane resistance this was accompanied by a depolarization rather than the hyperpolarization that one would expect. A possible explanation of this is that the fibres were

Fig.48 The responses of two cells (I & II) in muscle bundle 34 to increasing concentrations of GABA (50 µl volume) and also to muscimol. The recording electrode contained 3 M KCl.


(I) E_m =57mV
IO^{-6}M GABA
IO^{-5}M GABA
IO^{-4}M GABA
IO^{-3}M GABA
IO^{-3} MUSCIMOL
(II) E_m =53mV
IO^{-5}M GABA
IO^{-4}M GABA
IO^{-3}M GABA
20mV
I min

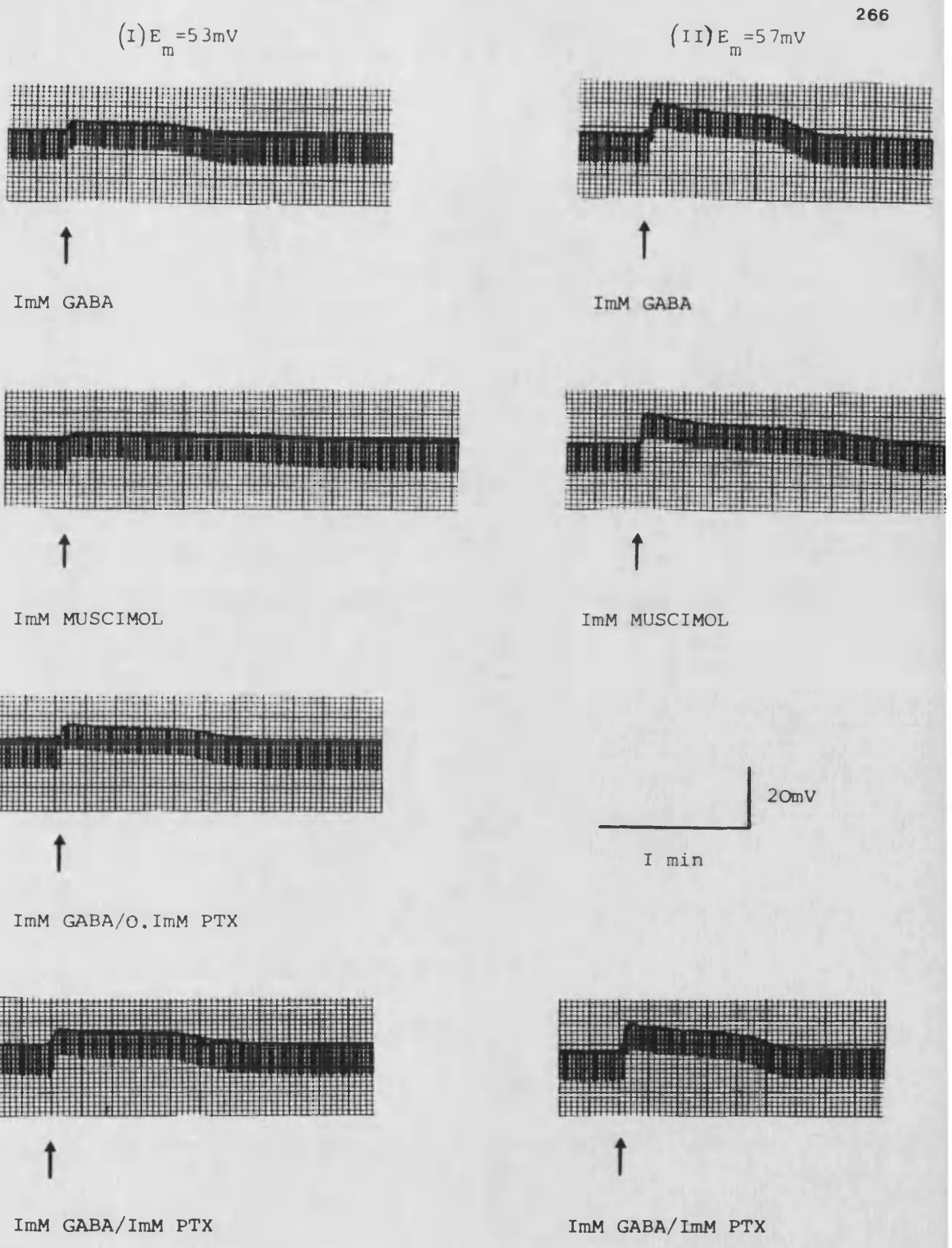
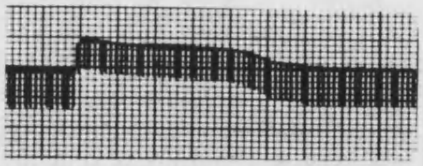
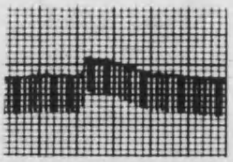


Fig.49 The responses of two cells (I & II) in muscle bundle 34 to GABA, muscimol and picrotoxin (50 µl volume). The recording electrode contained 3 M KCl.

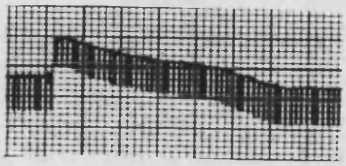
Fig.50 The responses of a single cell in muscle bundle 34 to GABA (50 µl volume) in the presence and absence of diazepam and bicuculline. A control shows the effect of 1% ethanol which was the ethanol concentration of samples containing diazepam and bicuculline. The recording electrode contained 3 M KCl.



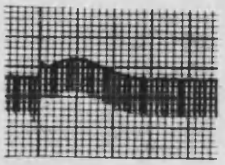
ImM GABA



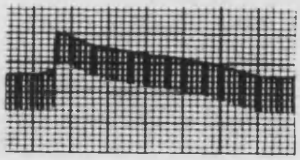
O.ImM GABA



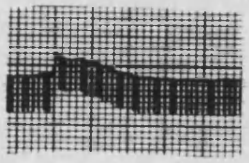
ImM GABA
+ O.ImM DZ.



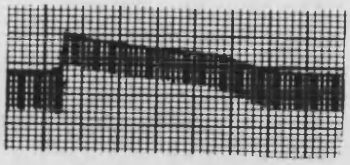
O.ImM GABA
+ O.ImM BIC.



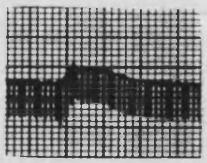
ImM GABA



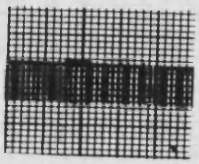
O.ImM GABA



ImM GABA
+ O.ImM DZ.



O.ImM GABA
+ O.ImM BIC.



I% ETHANOL

E_m=5 3mV

becoming loaded with chloride, in part from the high KCl concentration in the electrode inside the cell. Hence when GABAergic chloride channels were opened, chloride flowed out of, rather than into, the cell, causing a depolarization rather than a hyperpolarization. One way of reducing this effect is to use a non-chloride electrolyte. Therefore in all subsequent experiments 2 M potassium acetate was used in the recording electrode.

On changing the electrolyte from KCl to potassium acetate (Figs.51-56), though depolarizations were still observed in some cells in response to GABA (Fig.54), other cells showed either a hyperpolarization (Fig.55) or no change in membrane potential (Fig.52). In some experiments GABA was applied directly (50 µl) by pipette rather than by perfusion but when these two methods of application were compared in the same cell there was no significant difference in the responses (Fig.51). Muscimol (1 mM) caused the same reduction in resistance change as 1 mM GABA and the effect took longer to wash out than that of GABA, as was observed using the KCl electrode. Furthermore GABA and muscimol appear to be acting on the same channels because perfusion of the muscle with 1 mM muscimol immediately after perfusion with 1 mM GABA did not result in any additional change in resistance (Fig.52).

Isoguvacine and 3-aminopropane sulphonate (3APS) are potent mammalian central GABA agonists (Greenlee et al., 1978b; Williams & Risley, 1979). A

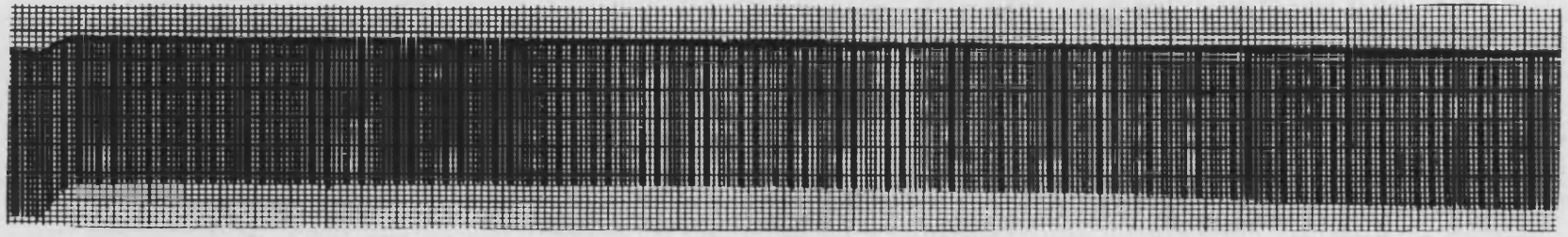
cell which depolarized in response to 1 mM GABA also depolarized in response to 1 mM isoguvacine but 3APS caused a hyperpolarization. Following the addition of 1 mM isoguvacine, 1 mM GABA only gave a slight additional depolarization but 3APS still caused a hyperpolarization. Furthermore, following a 3APS-induced hyperpolarization, 1 mM GABA still produced a full depolarization and 1 mM isoguvacine did not cause any further depolarization (Fig.53). Therefore it appears that isoguvacine acts on the same channels as GABA but that 3APS does not.

No consistent effects on GABA responses were observed with diazepam. Diazepam alone caused a slight hyperpolarization but this was also obtained with ethanol at the same concentration as in the diazepam. The only observed effect of diazepam was that following the depolarization and reduction in resistance caused by 0.1 mM GABA in the presence of 0.1 mM diazepam the cell took longer to return to its resting state than when exposed to GABA alone. Also on some occasions an additional depolarization was observed on washing off the GABA and diazepam (Fig.54).

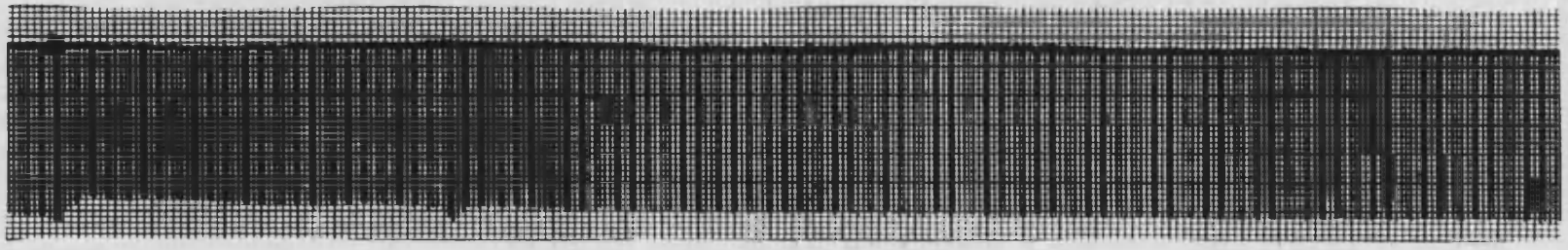
Whether a cell responded to GABA by depolarizing or by hyperpolarizing, the response was reduced by 1 mM bicuculline methiodide (Fig.55). The effect of 1 mM GABA was completely blocked by 0.5 mM picrotoxin in some cells (Fig.56), and when this occurred no further GABAergic responses could be

obtained in the preparation following the application of picrotoxin, despite extensive washing, implying that the effect of picrotoxin is irreversible.

Fig.51 The responses of a single cell in muscle bundle 34 to 1 mM GABA either bath applied for the duration shown (top trace) or as a 50 µl volume (bottom trace). The recording electrode contained 2 M potassium acetate.

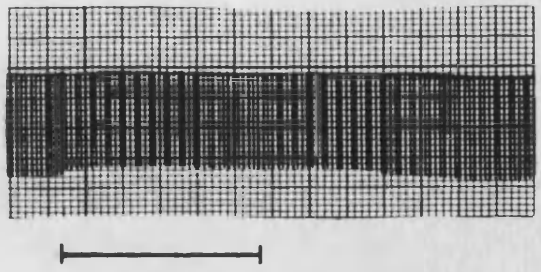


1mM GABA

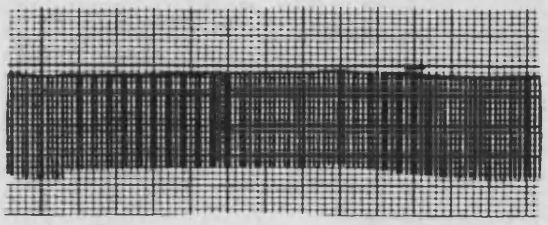


1mM GABA

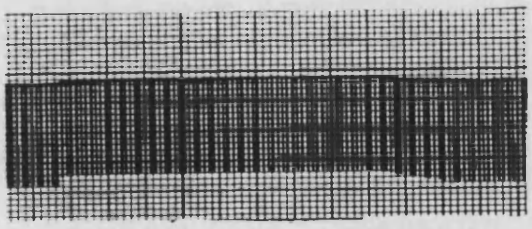
46mV E_m=65mV



1mM GABA



1mM MUSCIMOL



1mM GABA 1mM MUSCIMOL

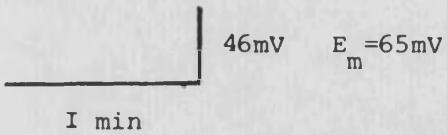
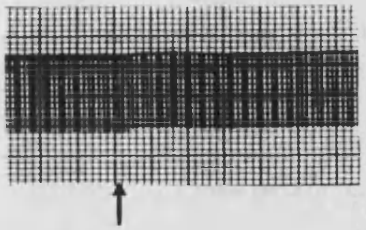
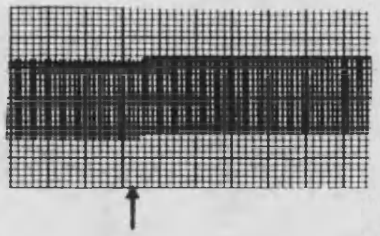


Fig.52 The responses of a single cell in muscle bundle 34 to GABA and muscimol bath applied as indicated. The recording electrode contained 2 M potassium acetate.

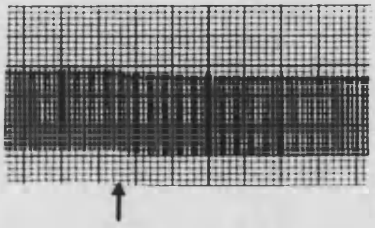
Fig.53 The responses of a single cell in muscle bundle 34 to GABA, isoguvacine and 3APS applied as 50 µl volumes. The recording electrode contained 2 M potassium acetate.



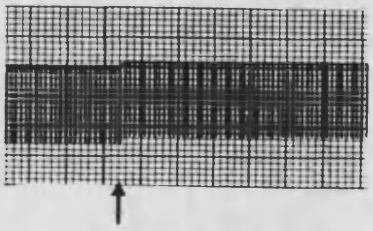
ImM GABA



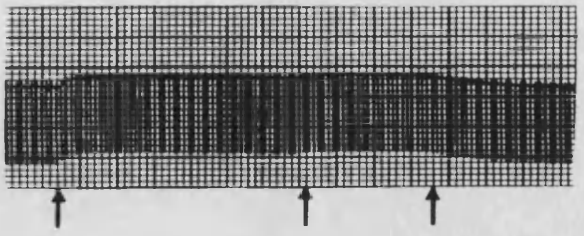
ImM ISOGUV.



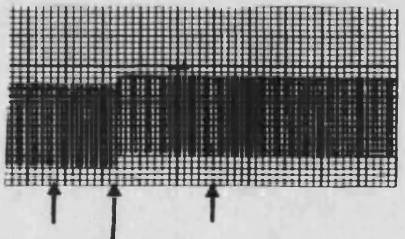
ImM 3APS



ImM GABA

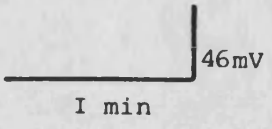


ImM ISOGUV. ImM GABA ImM 3APS



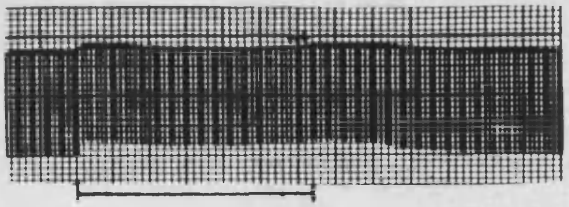
ImM 3APS ImM ISOGUV.

ImM GABA

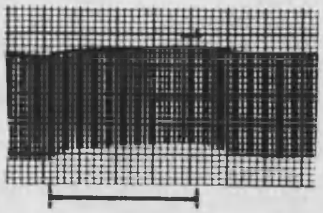


E_m=57mV

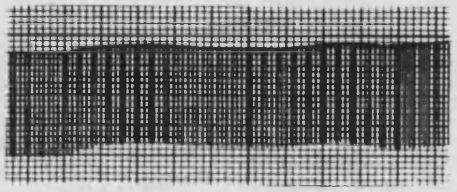
Fig.54 The responses of a single cell in muscle bundle 34 to GABA in the presence and absence of diazepam bath applied as indicated. The recording electrode contained 2 M potassium acetate.



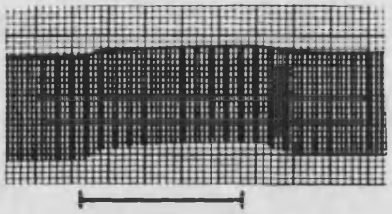
ImM/O.ImM DZ.
GABA



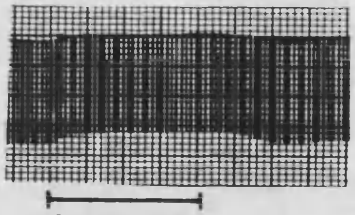
O.ImM GABA



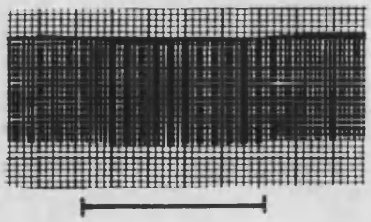
O.ImM GABA
+ O.ImM DZ.



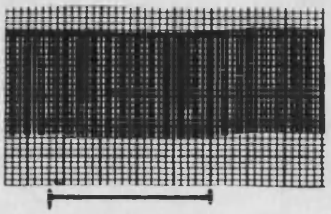
O.ImM GABA



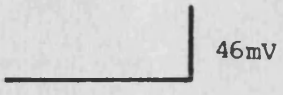
O.ImM GABA
+ O.ImM DZ.



O.ImM DZ.



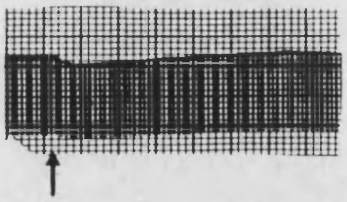
I% ETHANOL



E_m=65mV

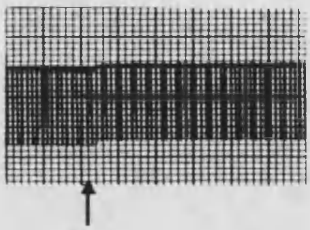
Fig.55 The responses of three cells (I, II & III) in muscle bundle 34 to GABA in the presence and absence of bicuculline methiodide bath applied as indicated. The recording electrode contained 2 M potassium acetate.

(I) E_m =70mV

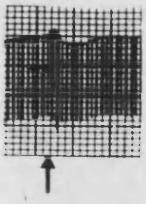


ImM GABA

(II) E_m =57mV

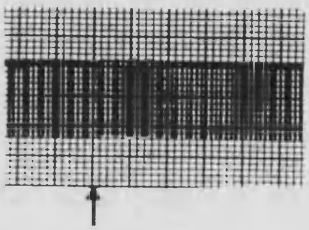


ImM GABA



ImM GABA

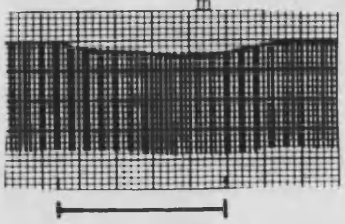
ImM BIC.



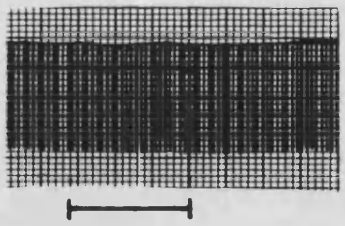
ImM GABA

ImM BIC.

(III) E_m =65mV

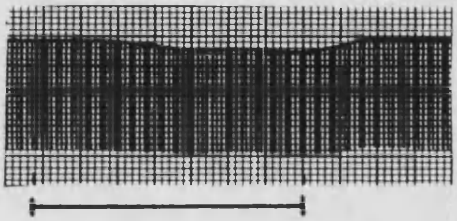


O.ImM GABA

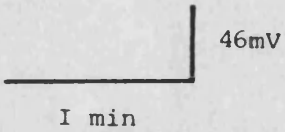


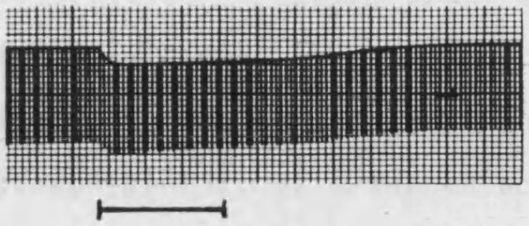
O.ImM GABA

+ ImM BIC.

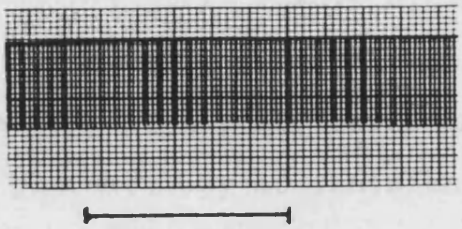


O.ImM GABA

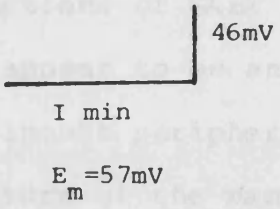




1mM GABA



1mM GABA
+ 0.5mM PTX



E_m=57mV

Fig.56 The responses of a single cell in muscle bundle 34 to GABA and picrotoxin bath applied as indicated. The recording electrode contained 2 M potassium acetate.

(1974). These workers extracted a proteolipid from shrimp muscle that bound [^{14}C]GABA with a K_D of $8x10^{-6}$ M. At 10^{-4} M, bicuculline was the most potent displacing ligand while picrotoxin (10^{-4} M) and the mammalian GABA agonist, muscimol ($4x10^{-4}$) were equally potent. However such proteolipids are difficult to work with and no further work has been published in this area.

The first invertebrate binding studies on putative GABA receptors were performed in crayfish muscle by Olsen et al. (1978) who demonstrated specific binding of [^{3}H]dihydropicrotoxin and the same workers went on to demonstrate specific, saturable Na^{+}-independant binding of [^{3}H]muscimol (K_D 9 nM) in the same tissue (Meiners et al., 1979). The [^{3}H]muscimol binding was sensitive to 3APS, isoguvacine and GABA, but not picrotoxin while the [^{3}H]dihydropicrotoxin binding was unaffected by GABA. The authors suggest that this is consistent with the idea that picrotoxin blocks GABA synaptic responses at a site distinct from the GABA receptor and possibly involving the chloride ion channel.

It was not until 1983 that a similar binding activity was reported in insect tissue. Abalis et al. (1983) detected the binding of the benzodiazepine [^{3}H]flunitrazepam (FNZP) to housefly thoracic membranes (though they could not detect GABA or muscimol binding in the same preparations). This binding was enhanced by GABA in a dose-dependent manner, optimal at 10^{-8} M GABA,

which in turn was blocked by the GABA antagonist, bicuculline at 1.7×10^{-5} M. Though this preparation would contain a proportion of thoracic ganglionic membranes, the authors attribute the observed binding to muscle membranes. More recently, using membranes prepared from the whole thorax and abdomen of the housefly, Cohen & Casida (1986) have reported the high affinity binding (K_D 210 nM) of the cage convulsant [^{35}S]TBPS. Working with 2 nM [^{35}S]TBPS, picrotoxinin is not very potent in displacing the ligand (IC_{50} 328 μM) while the IC_{50}s for displacement by diazepam and the barbiturate, pentobarbital, are 15 μM and 125 μM respectively. The binding of [^{35}S]TBPS at low concentrations was enhanced by GABA (1-100 μM) but as the [^{35}S]TBPS concentration was increased the effect disappeared. This could possibly result from a heterogeneous population of [^{35}S]TBPS binding sites in the membrane preparation (which is from a very heterogeneous tissue source and would possess both muscle and neuronal membranes), only one of which is affected by GABA. Though the GABA linkage described in this study is interesting, such work should perhaps be viewed carefully in the light of the report of Abalis et al. (1985a) that Torpedo electroplax bind [^{35}S]TBPS even though they are known not to possess any GABA receptors.

In short, there is ample electrophysiological evidence that insect leg muscles possess GABA receptors though it is not known whether these receptors are as

complex as their mammalian central $GABA_A$ counterparts (Usherwood, 1978). We also have biochemical evidence for the presence of GABA receptors on the tail muscle of another arthropod, the crayfish (Olsen et al., 1978; Meiners et al., 1979). However, for reasons of availability of tissue, the only biochemical studies on insect muscle GABA receptors have been done on whole housefly thoracic and abdominal preparations, which are not known to have any GABAergic innervation. Though these tissues do not exhibit GABA or muscimol binding, we have secondary evidence for GABAergic activity by the presence of GABA-linked benzodiazepine (Abalis et al., 1983) and cage-convulsant (Cohen & Casida, 1985) binding activities.

Because [^{3}H]muscimol and [^{3}H]FNZP binding assays had been developed for locust central nervous tissue (Chapter 2), it was decided to measure the binding of these ligands to a locust muscle preparation. The locust flight muscle used for these studies is comparable with the preparation of Abalis et al. (1983) who used whole housefly thorax.

Abalis et al. (1983) could not detect [^{3}H]GABA or [^{3}H]muscimol binding in housefly thoracic membranes. Using locust flight muscle membranes, the specific binding of [^{3}H]muscimol was only 5% of the total binding (Fig.44) and if this binding is real, any GABA receptors on these muscles have a very low affinity for [^{3}H]muscimol. Clearly locust flight muscle membranes do

not exhibit high affinity [^{3}H]muscimol binding comparable with locust central nervous tissue (chapter 2). However the same muscle membrane preparation did exhibit specific binding of [^{3}H]FNZP and the order of potency of the ligands tested in displacing this binding (Table 18) was the same as reported by Abalis et al. (1983), though they were all more potent, and indeed the actual potencies of the ligands in displacing [^{3}H]FNZP binding from locust muscle membranes are more similar to locust ganglionic [^{3}H]FNZP binding (2.3.2.5.5) than the housefly binding (Abalis et al., 1983). The binding of [^{3}H]FNZP in all three of these tissues is similar in being more potently displaced by the mammalian peripheral benzodiazepine, Ro5-4864, than by the central ligand, clonazepam. Interestingly, whereas it is necessary to add $CaCl_2$ to locust ganglionc membranes to obtain maximal [^{3}H]FNZP binding (2.3.2.3), the addition of $CaCl_2$ and $MgCl_2$ in place of 1 mM EGTA to locust muscle membranes did not result in an increase in binding and indeed the level of binding became more variable. The concentration of calcium in the muscle and the membranes prepared from it was very similar (Fig.45) implying little of the calcium is removed during the membrane preparation. However any free calcium would be chelated by the EGTA and therefore it appears that the muscle [^{3}H]FNZP binding may differ in its ionic requirements from the ganglionic binding. Evidently benzodiazepines do bind to locust flight muscle, but the

only evidence, to date, that this is linked to GABAergic activity, is that of Abalis et al. (1983), as we have no evidence for muscimol binding activity in the same tissue.

An electrophysiological study was made on the extensor tibiae of the locust femur, which is known to possess GABA receptors. The principal problem which was encountered was that of E_{Cl} being close to the membrane potential, as discussed in the results section, with the result that following a single GABA-induced hyperpolarization, no further GABA-evoked changes could be detected in the membrane potential. As a result it was not possible to use the technique of iontophoresis because it was thought that the same problem would arise, and therefore it was necessary to measure resistance changes across the muscle membrane in response to bath applied GABA. Once this system was set up GABAergic responses were observed in muscle bundle 34 of the extensor tibiae and it was possible to make a preliminary study of the pharmacology of these responses.

The GABA responses were mimicked by both muscimol and isoguvacine. The binding of [^{3}H]FNZP to housefly thoracic membranes described by Abalis et al. (1983) was enhanced by muscimol and isoguvacine while 3APS was inactive, whereas the binding of [^{3}H]muscimol to crayfish muscle (Meiners et al., 1979) is sensitve to GABA, isoguvacine and 3APS. Neither muscimol or 3APS had

any effect on the binding of [^{35}S]TBPS to housefly membranes (Cohen & Casida, 1985). 3APS did affect the locust muscle but these effects did not appear to be related to effects mediated by GABA. Furthermore, GABA and isoguvacine caused a depolarization while 3APS caused a hyperpolarization and since the rationale for a GABAergic depolarization is loading of the muscle with chloride ions, it may be that 3APS was not acting on chloride channels.

Bicuculline always affected the locust ET GABA responses, in agreement with Usherwood & Cull-Candy (1975). More recently Chalmers et al. (1985) have reported that GABA-evoked responses in crayfish dactyl abductor muscle are reduced in the presence of 0.1 mM bicuculline, though the IC_{50} for bicuculline was only 3 mM in the [^{3}H]muscimol binding study of Meiners et al. (1979) in crayfish muscle. However it is widely accepted that insect muscle GABA receptors are sensitive to bicuculline (Usherwood, 1978) and in this respect they seem different from the insect central GABA receptors, as discussed in chapter 2.

Picrotoxin completely blocked the GABA response in some ET cells, while other cells exhibited GABA responses which were insensitive to picrotoxin. A problem in assessing these data is that high concentrations of GABA had to be used in some cells and it is possible that these concentrations were too high to see any block by picrotoxin. However, 0.5 mM

picrotoxin completely blocked the response of some cells to 1mM GABA whereas 1mM picrotoxin had no effect on the responses of other cells to 1mM GABA. Thus the GABA receptors on the muscle cells appear to be heterogeneous with respect to their sensitivity to picrotoxin, in agreement with previous workers (Usherwood, 1978).

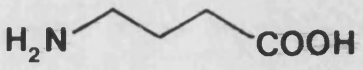
No consistant effect on GABA-evoked responses was observed with the benzodiazepine, diazepam. This again is not conclusive because of the high concentrations of GABA that were used; there is a possibility that at lower (non-saturating) GABA concentrations an effect of diazepam would be seen. However no evidence was obtained in this study for the GABA receptors on locust ET muscle being linked to benzodiazepine receptors.

In conclusion, the extensor tibiae muscle has been shown, electrophysiologically, to possess GABA receptors which are sensitive to muscimol, isoguvacine and bicuculline. A proportion of the receptors are blocked by picrotoxin, while 3APS does not appear to act via GABA receptors. Caution must be exercised in interpreting these data, though, because it is not known what proportions of the receptors are junctional and extrajunctional. However, this preparation could be used to elucidate the pharmacology of the muscle receptors more fully, and to determine whether insect muscle receptors are complexes of interacting binding sites, comparable with mammalian

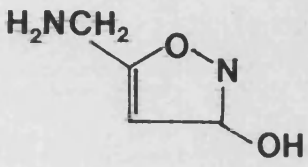
$GABA_A$ receptors, or whether they are ubiquitously simple receptors associated only with a chloride ion channel.

APPENDIX 2

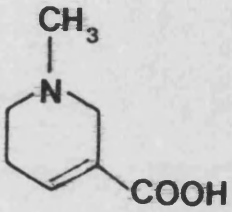
THE STRUCTURES OF COMPOUNDS USED IN THE STUDY



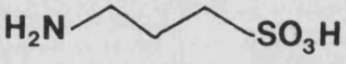
GABA



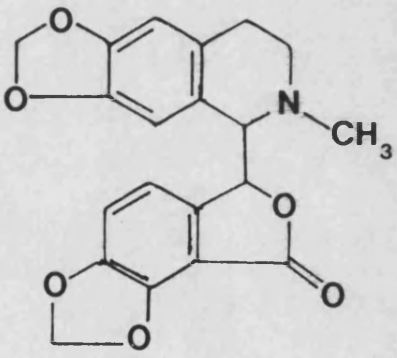
MUSCIMOL



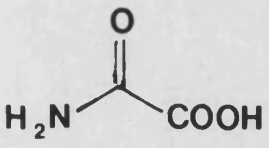
ISOGUVACINE



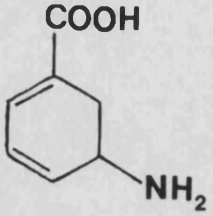
3APS



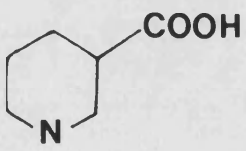
BICUCULLINE



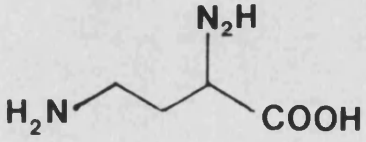
AOAA



GABACULLINE

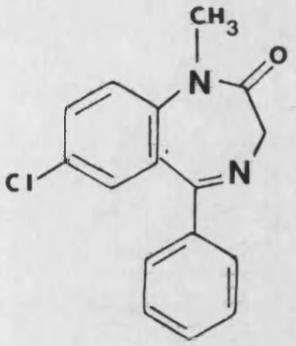


NIPECOTIC ACID

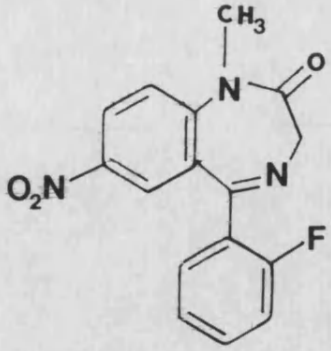


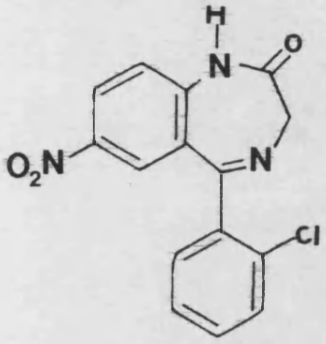
2,4-DABA

DIAZEPAM

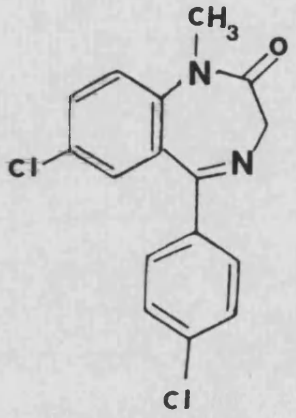


FLUNITRAZEPAM

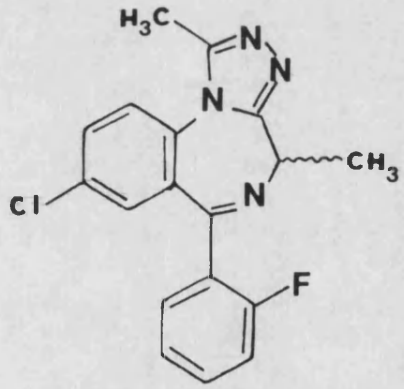




CLONAZEPAM

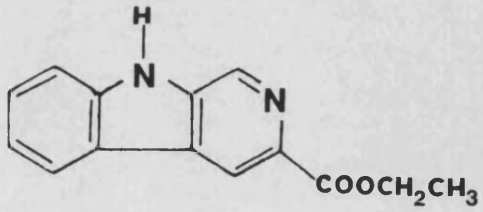


Ro5-4864

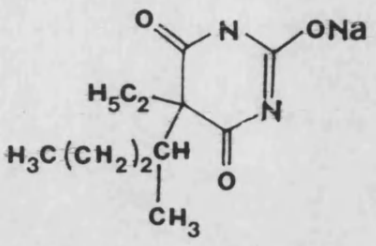


RoII-5073 (+)

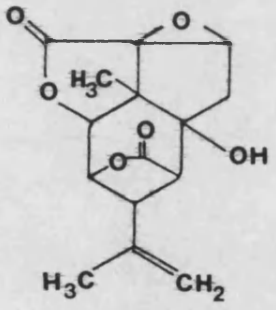
RoII-523I (-)



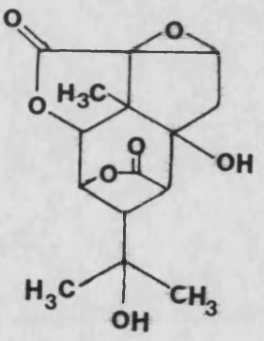
Ro15-2538



SODIUM PENTOBARBITAL

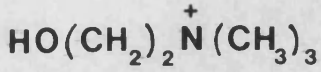


PICROTOXININ

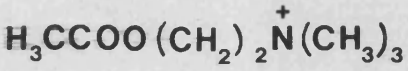


PICROTIN

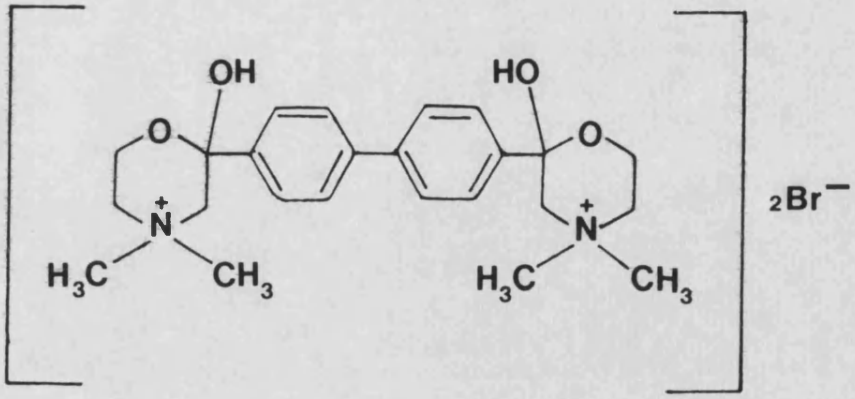
PICROTOXIN



CHOLINE



ACETYLCHOLINE



HEMICHOLINIUM-3

PUBLICATIONS

Lunt, G. G., Robinson, T. N., Miller, T., Knowles, W. P. & Olsen, R. W. (1984) [^{3}H]Muscimol binding to GABA receptors in insect CNS. Abst. 14th Ann. Meet. Neurosci. 10, 668.

Robinson, T. N., Lunt, G. G. & Olsen, R. W. (1984) Preliminary characterization of a GABA receptor in insect ganglia, in "Regulation of Transmitter Function: Basic and Clinical Aspects" (Eds. Vizi, E. S. & Magyar, K.), Akademiai Kiado, Budapest, Hungary, 276.

Lunt, G. G., Robinson, T. N., Miller, T., Knowles, W. P. & Olsen, R. W. (1985) The identification of GABA receptor binding sites in insect ganglia. Neurochem. Int. 7, 751-754.

Robinson, T. N., Lunt, G. G., Battersby, M. K., Irving, S. N. & Olsen, R. W. (1985) Locust ganglia contain [^{3}H]flunitrazepam binding sites. Biochem. Soc. Trans. 13, 716-717.

Robinson, T. N., Lunt, G. G., Battersby, M. K. & Irving, S. N. (1985) Locust ganglia contain receptors for 1,3-dihydro-2H-1,4-benzodiazepine derivatives. Pest. Sci. 16, 697-698.

Lunt, G. G., MacAllen, D. & Robinson, T. N. (1985) Photoaffinity labelling of the benzodiazepine receptor of the GABA receptor complex in locust CNS. 15th Am. Soc. Neurosci. Abstr. II (1), 275.

Robinson, T. N., MacAllen, D., Lunt, G. G. & Battersby, M. K. (1986) The GABA receptor of insect CNS: characterization of a benzodiazepine binding site. J. Neurochem. in press.

Robinson, T. N. & Lunt, G. G. (1986) A rapid technique for the production of a locust synaptosomal preparation. Biol. Chem. Hoppe Seyler. 367 Suppl., 298.

Robinson, T. N. & Lunt, G. G. (1986) Choline uptake and release by a locust ganglionic synaptosomal preparation, in "Molecular Basis of Neural Function" (Eds. Tucek, S., Stipek, S., Stastny, F. & Krivanek, J.), Abstr. Sixth ESN Ann. Gen. Meet., 128.

Robinson, T. N. & Olsen, R. W. (1987) GABA, in "Comparative Invertebrate Neurochemistry" (Eds. Lunt, G. G. & Olsen, R. W.), Croom-Helm, England, in press.

REFERENCES

Abalis, I. M. & Eldefrawi, A. T. (1986) [^{3}H]Muscimol binding to a putative GABA receptor in honey bee brain and its interaction with avermectin B_{1a}. Pest. Biochem. Physiol. 25, 279-287.

Abalis, I. M., Eldefrawi, M. E. & Eldefrawi, A. T. (1983) Biochemical identification of putative GABA/benzodiazepine receptors in housefly thorax muscles. Pest. Biochem. Phys. 20, 39-48.

Abalis, I. M., Eldefrawi, M. E. & Eldefrawi, A. T. (1985a) Binding of GABA receptor channel drugs to a putative voltage-dependant Cl^- channel in Torpedo electric organ. Biochem. Pharmacol. 34, 2579-2582.

Abalis, I. M., Eldefrawi, M. E. & Eldefrawi, A. T. (1985b) High affinity stereospecific binding of cyclodiene insecticides and γ-hexachlorocyclohexane to γ-aminobutyric acid receptors of rat brain. Pest. Biochem. Pharmacol. 24, 95-102.

Abalis, I. M., Eldefrawi, A. T. & Eldefrawi, M. E. (1986) Actions of AVM_{Bla} on $GABA_A$ receptor and chloride channels in rat brain. Biochem. Toxicol. in press.

Abe, M. & Matsuda, M. (1983) On the existence of two GABA pools associated with newly synthesised GABA and with newly taken up GABA in nerve terminals. Neurochem. Res. 8, 563-573.

Ackerman, D. & Kutscher, F. (1910) Uber aporrhegmen. Z. Physiol. Chem. 69, 1265-1272.

Adrian, W. J. (1973) A comparison of a wet pressure digestion method with other commonly used wet and dry-ashing methods. Analyst 98, 213-216.

Aguilar, J. S. & Lunt, G. G. (1984) Cholinergic binding sites with muscarinic properties on membranes from the supraoesophageal ganglion of the locust (Schistocerca gregaria). Neurochem. Int. 6, 501-507.

Aprison, M. H., McBride, W. J. & Freeman, A. R. (1973) The distribution of several amino acids in specific ganglia and nerve bundles of the lobster. J. Neurochem. 21, 87-95.

Asano, T., Yamada, Y. & Ogasawara, N. (1983) Characterization of the solubilized GABA and benzodiazepine receptor from various regions of bovine brain. J. Neurochem. 40, 209-214.

Awapara, J., Landua, A. J., Fuerst, R. & Seale, B.

(1950) Free gamma-aminobutyric acid in brain. J. Biol. Chem. 187, 35-39.

Basile, A. S. & Skolnick, P. (1986) Subcellular localization of "peripheral-type" binding sites for benzodiazepines in rat brain. J. Neurochem. 46, 305-308.

Battelle, B. A., Kravitz, E. A. & Stieve, H. (1979) Neurotransmitter synthesis in Limulus ventral nerve photoreceptors. Experientia 35, 778-780.

Baxter, C. F. & Torralba, G. F. (1975) γ-Aminobutyric acid glutamate decarboxylase (L-glutamate 1-carboxy-lyase, E.C.4.1.1.15) in the nervous system of the cockroach, Periplaneta americana, 1. Regional distribution and properties of the enzyme. Brain Res. 84, 383-397.

Bazemore, A. K., Elliott, A. C. & Florey, E. (1957) Isolation of factor I. J. Neurochem. 1, 334-339.

Beadle, D. J. & Lees, G. (1986) Insect neuronal cultures - a new tool in insect neuropharmacology, in "Neuropharmacology and Pesticide Action" (Eds. Ford, M. G., Lunt, G. G., Reay, R. C. & Usherwood, P. N. R.), Ellis & Horwood, England, 423-444.

Beadle, D. J., Benson, J. A., Lees, G. & Neuman, R.

(1985) Flunitrazepam and pentobarbital modulate GABA responses of insect neuronal somata. J. Physiol. 2731, 273P.

Bennett, J. P. (1978) Methods in binding studies, in "Neurotransmitter Receptor Binding" (Eds. Yamamura, H. I., Enna, S. J. & Kuhar, M. J.), Raven Press, New York, 57-90.

Bennett, J. P. Jnr., Mulder, A. H. & Snyder, S. H. (1974) Neurochemical correlates of synaptically active amino acids. Life Sci. 15, 1045-1056.

Birdsall, N. J. M. & Hulme, E. C. (1976) Biochemical studies on muscarinic acetylcholine receptors. 27, 7-18.

Blaustein, M. P. & Hodgkin, A. L. (1969) The effect of cyanide on the efflux of calcium from squid axons. J. Physiol. 200, 497-527.

Blinderman, J-M., Maitre, M., Ossola, L & Mandel, P. (1978) Purification and some properties of L-glutamate decarboxylase from human brain. Eur. J. Biochem. 86, 143-152.

Bock, R. (1979) A Handbook of Decomposition Methods in Analytical Chemistry, Blackie, Glasgow.

Bolger, G.T., Weissman, B. A., Lueddens, H., Basile, A. S., Mantione, C. R., Barrett, J. E., Witkin, J. M., Paul, S. M. & Skolnick, P. (1985) Late evolutionary appearance of peripheral-type binding sites for benzodiazepines. Brain Res. 338, 366-370.

Bond, P. A. (1973) The uptake of γ-[^{3}H]aminobutyric acid by slices from various regions of rat brain and the effect of lithium. J. Neurochem. 20, 511-517.

Bowery, N., Price, G. W., Hudson, A. L., Hill, D. R., Wilkin, G. P. & Turnbull, M.J. (1984) GABA receptor multiplicity - visualization of different receptor types in the mammalian CNS. Neuropharmacol. 23, 219-231.

Bowling, A. C. & De Lorenzo, R. J. (1982) Micromolar affinity benzodiazepine receptors: identification and characterization in central nervous systems. Science 216, 1247-1250.

Bradford, H. F. (1970) Metabolic responses of synaptosomes to electrical stimulation: release of amino acids. Brain Res. 19, 239-247.

Bradford, H. F. (1986) Chemical neurobiology. W. H. Freeman & Co., New York, 229-242.

Bradford, H. F., Chain, E. B., Cory, H.T. & Rose, S. P.

R. (1969) Glucose and amino acid metabolism in some invertebrate nervous systems. J. Neurochem. 16, 969-978.

Bradford, H. F., Jones, D. G., Ward, H. K. & Booher, J. (1975) Biochemical and morphological studies of the short and long term survival of isolated nerve endings. Brain Res. 90, 245-259.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Braestrup, C. & Nielsen, M. (1983) Benzodiazepine receptors, in "Handbook of Pharmacology Vol.17." (Eds. Iversen, L.L., Iversen, S. D. & Snyder, S. H.), Plennum Publishing, 285-384.

Braestrup, C. & Squires, R. F. (1977) Specific benzodiazepine receptors in rat brain characterized by high-affinity [^{3}H]diazepam binding. Proc. Natl. Acad. Sci. USA 74, 3805-3809.

Braestrup, C & Squires, R. F. (1978) Pharmacological characterization of benzodiazepine receptors in the brain. Eur. J. Pharmacol. 48, 263-270.

Breer, H. (1981a) Characterization of synaptosomes from

the central nervous system of insects. Neurochem. Int. 3, 155-163.

Breer, H. (1981b) Comparative studies on cholinergic activities in the central nervous system of Locusta migratoria. J. Comp. Physiol. 141, 271-276.

Breer, H. (1982a) Properties of synaptosomes from the central nervous system of insects, in "Neuropharmacology of Insects" (Ciba Foundation Symposium 88), Pitman, London, 158-175.

Breer, H. (1982b) Uptake of [N-Me-^{3}H]choline by synaptosomes from the central nervous system of Locusta migratoria. J. Neurobiol. 13, 107-117.

Breer, H. & Heilgenberg, H. (1986) Neurochemistry of GABAergic activities in the central nervous system of Locusta migratoria. J. Comp. Physiol. A 157, 343-354.

Breer, H. & Jeserich, G. (1980) A microscale floatation technique for the isolation of synaptosomes from nervous tissue of Locusta migratoria. Insect Biochem. 10, 457-463.

Breer, H. & Knipper, M. (1984) Characterization of acetylcholine release from insect synaptosomes. Insect Biochem. 14, 337-344.

Brenner, S. & Horne, R. W. (1959) A negative staining method for high resolution electron microscopy of viruses. Biochim. Biophys. Acta 34, 103-110.

Bylund, D. B. (1980) Analysis of receptor binding data, in "Receptor Binding Techniques" (short course syllabus), Society for Neuroscience, Bethesda, 70-99.

Calcott, P. H. & Fatig, R. O. (1984) Avermectin modulation of GABA binding to membranes of rat brain, brine shrimp and a fungus, Mucor miehei. J. Antibiot. 37, 253-259.

Callec, J. J. (1974) Synaptic transmission in the central nervous system of insects, in "Insect Neurobiology" (Ed. Treherne, J. E.), North Holland, Amsterdam, 119-178.

Carlin, R. K. & Siekevitz, P. (1984) Characterization of Na^+-independant GABA and flunitrazepam binding in preparations of synaptic membranes and postsynaptic densities from canine cerebellum and cerebral cortex. J. Neurochem. 43, 1011-1017.

Carroll, P. T. & Goldberg, A. M. (1975) Relative importance of choline transport to spontaneous and potassium depolarized release of acetylcholine. J.

Neurochem. 25, 523-527.

Casalotti, S. O., Barnard, E. A. & Stephenson, F. A. (1986) Photoaffinity labelling of the purified $GABA_A$ receptor with [^{3}H]muscimol, in "Molecular Basis of Neural Function" (Eds. Tucek, S., Stipek, S., Stastag, F. & Krivanek, J.), Proc. Sixth ESN Gen. Meet., 208.

Cavalla, D. & Neff, N. H. (1985) Photoaffinity labelling of the $GABA_A$ receptor with [^{3}H]muscimol. J. Neurochem. 44, 916-921.

Chalmers, A. E., Miller, T. A. & Olsen, R. W. (1985) A pharmacological investigation of invertebrate GABA receptors. Pest. Sci. 16, 435-436.

Chalmers, A. E., Miller, T. A. & Olsen, R. W. (1986) The action of avermectin on crayfish nerve and muscle. Eur. J. Pharmacol. in press.

Chang, L-R. & Barnard, E. A. (1982) The benzodiazepine/GABA receptor complex: molecular size in brain membranes and in solution. J. Neurochem. 39, 1507-1518.

Chang, K-J., Jacobs, S. & Cuatrecasas, P. (1975) Quantitative aspects of hormone-receptor interactions of high affinity: Effect of receptor concentration and

measurement of dissociation constants of labelled and unlabelled hormones. Biochim. Biophys. Acta 406, 294-303.

Chapman, R. F. (1975) The Insects: Structure and Function. The English Universities Press Ltd., 515-542.

Chen, R. S. & Widner, B. (1968) Content and synthesis of GABA in the larval brain of Drosophila melanogaster. Experientia 24, 516-517.

Chude, O., Roberts, E. & Wu J-Y. (1979) Partial purification of Drosophila glutamate decarboxylase. J. Neurochem. 32, 1409-1415.

Cline, H. T., Nusbaum, M.P. & Kristan, W. B. (1985) Identified GABAergic inhibitory motor neurones in the leech central nervous system take up GABA. Brain Res. 384, 359-362.

Cohen, E. & Casida, J. E. (1986) Effects of insecticides and GABAergic agents on a housefly [^{35}S]t-butylbicyclophosphorothionate binding site. Pest. Biochem. Physiol. 25, 63-72.

Constanti, A. & Nistri, A. (1981) Differential effects of Na^+-free media on GABA- and muscimol-evoked conductance increases recorded from lobster muscle

fibres. Neuroscience 6, 1443-1453.

Cori, G. T., Slein, M. W. & Cori, C. F. (1948) Crystalline D-glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle. J. Biol. Chem. 173, 605-618.

Cory, H. T. & Rose, S. P. R. (1969) Glucose and amino acid metabolism in octopus optic and vertical lobes in vitro. J. Neurochem. 16, 979-988.

Cotman, C. W., Haycock, J. W. & White, W. F. (1976) Stimulus secretion coupling processes in brain: analysis of noradrenaline and GABA release. J. Physiol. (London) 254, 475-505.

Craelius, W. & Fricke, R. A. (1981) Release of [^{3}H]gamma-aminobutyric acid (GABA) by inhibitory neurones of the crayfish. J. Neurobiol. 12, 249-258.

DeBelleroche, J. S. & Bradford, H. F. (1972) Metabolism of beds of mammalian cortical synaptosomes: responses to depolarizing influences. J. Neurochem. 19, 585-602.

DeBelleroche, J. S. & Bradford, H. F. (1977) On the site of origin of transmitter amino acids released by depolarization of nerve terminals in vitro. J. Neurochem. 29, 335-343.

DeFeudis, F. V. (1979) Binding and iontophoretic studies

on centrally active amino acids - a search for physiological receptors. Int. Rev. Neurobiol. 21, 155-177.

DeFeudis, F. V. (1980) Binding studies with muscimol; relation to synaptic γ-aminobutyrate receptors. Neuroscience 5, 673-688.

Deisz, R. A., Dose, M. & Lux, H. D. (1984) The time course of GABA action on the crayfish stretch receptor: evidence for a saturable GABA uptake. Neurosci. Lett. 47, 245-250.

DeLorenzo, R. J., Burdette, S. & Holderness, J. (1981) Benzodiazepine inhibition of the calcium-calmodulin protein kinase system in brain membrane. Science 213, 546-549.

Deng, L., Ransom, R. W. & Olsen, R. W. (1986) [^{3}H]Muscimol photolabels the γ-aminobutyric acid receptor binding site on a peptide subunit distinct from that labelled with benzodiazepines. Biochem. Biophys. Res. Comm. in press.

De Robertis, E. & Fiszer de Plazas, M. (1974) Isolation of hydrophobic proteins binding neurotransmitter amino acids: γ-aminobutyric acid receptor of the shrimp muscle. J. Neurochem. 23, 1121-1125.

De Robertis, E., Pellegrino DeIraldi, A., Rodriguez DeLores Arnaiz, G. & Salganiceff, L. (1962) Cholinergic and non-cholinergic nerve endings in rat brain I. J. Neurochem. 9, 23-25.

Dixon, W. E. (1906) Brit. Med. J. 2, 1807.

Donnellan, J. F., Alexander, K. & Chendlik, R. (1976) The isolation of cholinergic nerve terminals from fleshfly heads. Insect Biochem. 6, 419-423.

Dowdall, M. J. & Whittaker, V. P. (1973) Comparative studies in synaptosome formation: the preparation of synaptosomes from head ganglia of squid. J. Neurochem. 20, 921-935.

Drexler, G. & Sieghart, W. (1984a) Properties of a high affinity binding site for [^{3}H]avermectin$_{Bla}$. Eur. J. Pharmacol. 99, 269-277.

Drexler, G. & Sieghart, W. (1984b) [^{35}S]t-Butylbicyclophosphorothionate and avermectin bind to different sites associated with the gamma-aminobutyric acid-benzodiazepine receptor complex. Neurosci. Lett. 50, 273-277.

Drexler, G. & Sieghart, W. (1984c) Evidence for

association of a high affinity avermectin binding site with the benzodiazepine receptor. Eur. J. Pharmacol. 101, 201-207.

Duce, I. R. & Scott, R. H. (1985) Actions of avermectin$_{B1a}$ on insect muscle. Br. J. Pharmacol. 85, 395-401.

Dwivedy, A. K. (1985) Cholinergic properties of pinched-off synaptic nerve endings from the central nervous system of the cockroach, Periplaneta americana. Pest. Sci. 16, 615-626.

Eccles, J. C. & Jaeger, J. C. (1958) The relationship between the mode of operation and the dimensions of the junctional regions at synapses and motor end organs. Proc. Roy. Soc. (London) 148, 38-56.

Edwardson, J. M., Phillips, N. I., Kirby, N. & Fowler, L. J. (1985) A monoclonal antibody to rabbit brain GABA transaminase. J. Neurochem. 44, 1679-1684.

Ehlert, F. J. (1986) 'Inverse agonists', cooperativity and drug action at benzodiazepine receptors. TIPS. 7, 28-32.

Eichinger, A. & Sieghart, W. (1984) Photoaffinity labelling of different benzodiazepine receptors at

physiological temperatures. J. Neurochem. 43, 1745-1748.

Eliott, J. R. (1904) On the action of adrenalin. J. Physiol. (London) 31, 20P.

Enna, S. J. & Snyder, S. H. (1975) Properties of GABA receptor binding in rat brain synaptic membrane fractions. Brain Res. 100, 81-97.

von Euler, U. S. (1985) The history of substance P, in "Neurotransmitters in Action" (Ed. Bousfield, D.), Elsevier Biomedical Press, Amsterdam - New York - Oxford, 143-150.

Filbin, M. T., Lunt, G. G. & Donnellan, J. F. (1983) Partial purification and characterization of an acetylcholine receptor with nicotinic properties from the supraoesophageal ganglion of the locust (Schistocerca gregaria). Eur, J. Biochem. 132, 151-156.

File, S. E. & Pellow, S. (1985) The anxiogenic action of Ro5-4864 in the social interaction test: effect of chlordiazepoxide, Ro15-1788 and CGS-8216. N.Sch's Arch. Pharmacol. 328, 225-228.

Fischer, J. B. & Olsen, R. W. (1986) Biochemical aspects of GABA/benzodiazepine receptor function, in "Benzodiazepine/GABA Receptors and Chloride Channels:

Structural and Functional Properties" (Eds. Olsen, R. W. & Ventner, J. C.), Alan R. Liss. 241-259.

Fisher , T. E., Tuchek, J. M. & Johnson, D. D. (1986) Comparison of methods for removal of endogenous GABA from brain membranes prepared for binding assays. Neurochem. Res. 11, 1-8.

Florey, E. (1954) An inhibitory and excitatory factor from mammalian brain and their action on a single sensory neurone. Arch. Intern. Physiol. 62, 33-53.

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) Proc. Int. Neurochem. Symp. 13, 185.

Fonnum, F. (1969) Isolation of choline esters from aqueous solutions by extraction with sodium tetraphenylboron in organic solvents. Biochem. J. 113, 291-298.

Fowler, L. J., Lovell, D. H. & John, R. A. (1983) Reaction of muscimol with 4-aminobutyrate aminotransferase. J. Neurochem. 41, 1751-1754.

Fox, P. M. & Larsen, J. R. (1972) Glutamic acid decarboxylase and the GABA shunt in the supraoesophageal ganglion of the honeybee, Apis mellifera. J. Insect Physiol. 18, 439-457.

Fritz, L.C., Wang, C.C. & Gorio, A.(1979) Avermectin$_{B1a}$ irreversibly blocks postsynaptic potentials at the lobster neuromuscular junction by reducing muscle membrane resistance. Proc. Natl. Acad. Sci. USA 76, 2062-2066.

Frontali, N. (1961) Activity of glutamic acid decarboxylase in insect nervous tissue. Nature 191, 178-179.

Frontali, N. (1964) Brain glutamic acid decarboxylase and synthesis of γ-aminobutyric acid in vertebrate and invertebrate species, in "Comparative Neurochemistry" (Ed. Richter, D.), Pergamon, Oxford, 185-192.

Frontali, N. & Pierantoni, R. (1973) Autoradiographic localisation of [^{3}H]GABA in the cockroach brain. Comp. Biochem. Physiol. 44A, 1369-1372.

Galper, J. B., Klein, W. & Catterall, W. A. (1977) Muscarinic acetycholine receptors in developing chick heart. J Biol. Chem. 252, 8692-8699.

Gavish, M. & Fares, F. (1985) Solubilization of peripheral benzodiazepine binding sites from rat kidney. J. Neurosci. 5, 2889-2893.

Gee, K. U. & Yamamura, H. I. (1982) Regional heterogeneity of benzodiazepine receptors at 37°C: an *in vitro* study in various regions of the brain. Life Sci. 31, 1939-1945.

Gehlert, D. R., Yamamura, H. I. & Wamsley, J. K. (1985) Autoradiographic localization of "peripheral type" benzodiazepine binding sites in rat brain, heart and kidney. N. Sch's. Arch. Pharm. 328, 454-460.

Gerschenfeld, H. M. (1973) Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. Phys. Rev. 53, 1-119.

Gibson, G. E. & Blass, J. P. (1982) Synaptosomes, in "Progress in Cholinergic Biology: Model Cholinergic Synapses" (Eds. Hannin, I. & Goldberg, M. A.), Raven Press, New York, 289-321.

Gordon, D., Zlotkin, E., & Kanner, B. (1982) Functional membrane vesicles from the nervous system of insects, 1. Na^+ and Cl^- dependent GABA transport. Biochem. Biophys. Acta 688, 229-236.

Gray, E. G. & Whittaker, V. P. (1962) The isolation of nerve endings from brain: an electron microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat. Lond. 96, 79-87.

Greenlee, D. V. & Olsen, R. W. (1979) Solubilization of gamma-aminobtyric acid receptor protein from mammalian brain. Biochem. Biopys. Res. Comm. 88, 380-387.

Greenlee, D. V., Van Ness, P. C. & Olsen, R. W. (1978a) Endogenous inhibitor of GABA binding in mammalian brain. Life Sci. 22, 1653-1662.

Greenlee, D. V., Van Ness, P. C. & Olsen, R. W. (1978b) Gamma-aminobutyric acid binding in mammalian brain: Receptor-like specificity of sodium-independant sites. J. Neurochem. 31, 933-938.

Griffiths, R., Larsson, O. M., Allen, I. C. & Schousboe, A. (1986) Reassesment of β-alanine as a selective inhibitor of GABA uptake by glia. Biochem. Soc. Trans. in press.

Haefely, W. (1984) Benzodiazepine interactions with GABA receptors. Neurosci. Lett. 47, 201-206.

Hall, Z. W. & Kravitz, E.A. (1967a) The metabolism of γ-aminobutyric acid (GABA) in the lobster nervous system. I. GABA-glutamate transaminase. J. Neurochem. 14, 45-54.

Hall, Z. W. & Kravitz, E.A. (1967b) The metabolism of γ-aminobutyric acid (GABA) in the lobster nervous system.

II. Succinic semialdehyde dehydrogenase. J. Neurochem. 14, 55-61.

Hamilton, E. I., Minski, M. J. & Cleary, V. (1967) The loss of elements during the decomposition of biological materials with special reference to arsenic, sodium, strontium and zinc. Analyst 92, 257-259.

Haring, P., Stahli, C., Schoch, P., Takacs, B., Staehelin, T. & Mohler, H. (1985) Monoclonal antibodies reveal structural homogeneity of γ-aminobutyric acid/benzodiazepine receptors in different brain regions. Proc. Natl. Acad Sci. USA 82, 4837-4841.

Havoundjian, H., Cohen, R. M., Paul, S. M. & Skolnick, P. (1986) Differential sensitivity of "central" and "peripheral" type benzodiazepine receptors to phospholipase A_2. J. Neurochem. 46, 804-811.

Haycock, J. W., Levy, W. B., Denner, L. A. & Cotman, C. W. (1978) Effects of elevated $[[K^+]_o]$ on the release of neurotransmitters from cortical synaptosomes: efflux or secretion? J. Neurochem. 30, 1113-1125.

Hebebrand, J., Friedl, W., Lentes, K. L. & Propping, P. (1986a) Qualitative variation of photolabelled benzodiazepine receptors in different species. Neurochem. Int. 8, 267-271.

Hebebrand, J., Friedl, W., Unverzagt, B. & Propping, P. (1986b) Benzodiazepine receptors in avian brain. J. Neurochem. 47, 790-793.

Hemsworth, B. A. & Morris, D. (1964) A comparison of the N-alkyl group specificity of choline acetyltransferase from different species. J. Neurochem. 11, 793-803.

Hill, A. V. (1910) The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves. J. Physiol. (London) 40, iv-vii.

Hill, D. R., Bowery, N. G. & Hudson, A. L. (1984) Inhibition of $GABA_B$ binding by guanyl nucleotides. J. Neurochem. 42, 632-657.

Hirsch, J. D., Garrett, K. M. & Beer, B. (1985) Heterogeneity of benzodiazepine binding sites: a review of recent research. Pharmacol. Biochem. Behav. 23, 681-685.

Horne, R. W. & Whittaker, V. P. (1962) The use of the negative staining method for the electron microscopic study of subcellular particles from animal tissues. Z. Zellforsch. 58, 1-16.

Horwitz, I. S. & Orkand, R. K. (1979) GABA inactivation

at the crayfish neuromuscular junction. J. Neurobiol. 11, 447-458.

Hoyle, G. (1953) Potassium ions and insect nerve muscle. J. Exp. Biol. 30, 121-135.

Hoyle, G. (1978) Distributions of nerve and muscle fibre types in locust jumping muscle. J. Exp. Biol. 73, 205-233.

Hue, B., Gabriel, A & Le Patezour, A. (1982) Localization of [^{3}H]GABA accumulation in the sixth abdominal ganglion of the cockroach, Periplaneta americana. J. Insect Physiol. 28, 753-759.

Huggins, A. K., Rick, J. T. & Kerkut, G. A. (1967) A comparative study of the intermediate metabolism of L-glutamate in muscle and nervous tissue. Comp. Biochem. Physiol. 21, 23-30.

Irving, S. N. & Miller, T. A. (1980) L-Glutamate and aspartate as possible transmitters at 'fast' and 'slow' neuromuscular junctions in body-wall muscles of Musca larvae, in "Insect Neurobiology and Pesticide Action (Neurotox '79)", London Society of Chemical Industry, 227-233.

Isaac, R. A. & Johnson, W. C. (1975) Collaborative study

of wet and dry ashing techniques for the elemental analysis of plant tissue by atomic absorption spectrophotometry. J. Ass. Analyt. Chem. 58, 436-442.

Iversen, L. L. (1971) Role of transmitter uptake mechanisms in synaptic transmission. Br. J. Pharmacol. 41, 571-586.

Iversen, L. L. (1979) The chemistry of the brain, in "The Brain, a Scientific American Book", W. H. Freeman & Co., San Francisco, 70-83.

Iversen, L. L. & Bloom, F. E. (1972) Studies of the uptake of [^{3}H]GABA and [^{3}H]glycine in slices and homogenates of rat brain and spinal cord by electron microscopic autoradiography. Brain Res. 41, 131-143.

Iversen, L. L. & Johnstone, G. A. R. (1971) GABA uptake in rat central nervous system: comparison of uptake in slices and homogenates and the effects of some inhibitors. J. Neurochem. 18, 1939-1950.

Iversen, L. L. & Kelly, J. S. (1975) Uptake and metabolism of γ-aminobutyric acid by neurones and glial cells. Biochem. Pharmacol. 24, 933-938.

Iversen, L. L. & Kravitz, E. A. (1966) Uptake of γ-aminobutyric acid (GABA) in a lobster nerve-muscle

preparation. Fed. Proc. 25, 714.

Iversen, L. L. & Kravitz, E. A. (1968) The metabolism of γ-aminobutyric acid (GABA) in the lobster nervous system: uptake of GABA in nerve-muscle preparations. J. Neurochem. 15, 609-620.

Iversen, L. L. & Neal, M. J. (1978) The uptake of [^{3}H]GABA by slices of rat cerebral cortex. J. Neurochem. 15, 1141-1149.

John, R. A. & Fowler, L. J. (1976) Kinetic and spacial properties of rabbit brain 4-aminobutyrate aminotransferase. Biochem. J. 155, 645-651.

Johnson, M. K. (1960) The intracellular distribution of glycolytic and other enzymes in rat brain homogenates and mitochondrial preparations. Biochem. J. 77, 610-618.

Johnson, G. A. R., Kennedy, S. M. E. & Lodge, D. (1978) Muscimol uptake, release and binding in rat brain slices. J. Neurochem. 31, 1519-1524.

Johnson, R. W., Tallman, J. F., Squires, R. & Yamamura, H. I. (1983) Benzodiazepine receptor heterogeneity and regulation, in "Anxiolytics: Neurochemical, Behavioural and Clinical Perspectives" (Eds. Malick, J. B., Enna, S. J. & Yamamura, H. I.), Raven Press, New York, 93-112.

Jones, D. G. (1971) The negative staining of Octopus synaptosomes. Brain Res. 29, 378-382.

Jones, D. G. (1972) On the ultrastructure of the synapse: the synaptosome as a morphological tool!, in "The Structure and Function of Nervous Tissue Vol. 6" (Ed. Bourne, G. H.), New York Academic Press, 81-129.

Jones, D. G. (1975) Synapses and synaptosomes. Chapman & Hall, London.

Jope, R. S. (1979) High affinity choline transport and acetyl CoA production in brain and their roles in the regulation of acetycholine synthesis. Brain Res. Rev. 1, 313-344.

Kanner, B. I. (1978) Active transport of γ-aminobutyric acid by membrane vesicles isolated from rat brain. Biochemistry 17, 1207-1211.

Karobath, M. & Supavilai, P. (1982) Distinction of benzodiazepine agonists from antagonists by photoaffinity labelling of benzodiazepine receptors in vitro. Neurosci. Lett. 31, 65-69.

Karobath, M. & Supavilai, P. (1985) Interaction of benzodiazepine receptor agonists and inverse agonists

with the GABA/benzodiazepine receptor complex. Pharmacol. Biochem. Behav. 23, 671-674.

Kass, I. S., Wang, C. C., Walrond, J. P. & Stretton, A. O. W. (1980) Avermectin$_{Bla}$, a paralysing antihelminthic that affects interneurones and motorneurones in Ascaris. Proc. Natl. Acad. Sci. USA 77, 6211-6215.

Kirkness, E. F. & Turner, A. J. (1986a) The γ-aminobutyrate/benzodiazepine receptor from pig brain. Biochem. J. 233, 265-270.

Kirkness, E. F. & Turner, A. J. (1986b) Proteolytic degradation of the GABA-benzodiazepine receptor complex, in "Molecular Basis of Neural Function" (Eds. Tucek, S., Stipek, S., Stastny, F. & Krivanek, J.), Proc. Sixth ESN Gen. Meet., 206.

Klepner, C. A., Lippa, A. S., Benson, D. I., Sano, M. C. & Beer, B. (1979) Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. Pharmacol. Biochem. Behav. 11, 457-462.

Kravitz, E. A. (1967) Acetylcholine, gamma-aminobutyric acid and glutamic acid: physiological and chemical studies related to their role as neurotransmitter agents, in "The Neurosciences: a Study Program" (Eds. Quarton, G. C., Melnechuk, T. & Schmitt, F. O.),

Rockerfeller Univ., New York, 433-444.

Kravitz, E. A., Potter, D. D. & Van Gelder, N. M. (1962) Gamma-aminobutyric acid and other blocking substances extracted from crab muscle. Nature 194, 382-383.

Kravitz, E. A., Molinoff, P. B. & Hall, Z. W. (1965) A comparison of the enzymes and substrates of gamma-aminobutyric acid metabolism in lobster excitatory and inhibitory axons. Proc. Natl. Acad. Sci. USA 54, 778-782.

Kravitz, E. A., Iversen, L. L., Otsuka,M. & Hall, Z. W. (1968) Gamma-aminobutyric acid in the lobster nervous system: release from inhibitory nerves and uptake into nerve-muscle preparations, in "Structure and Function of Inhibitory Neuronal Mechanisms" (Eds. von Euler, C., Skoglund, S. & Soderberg, U.), Pergamon, Oxford, 371-376.

Krieger, D. T. (1983) Brain peptides: what, where and why? Science 222, 975-985.

Krogsgaard-Larsen, P., Hjeds, H., Curtis, D. R., Lodge, D. & Johnstone, G. A. R. (1979) Dihydromuscimol, thiomuscimol and related heterocyclic compounds as GABA analogues. J. Neurochem. 32, 1717-1724.

Kuhar, M. J. (1973) Neurotransmitter uptake: a tool in identifying transmitter-specific pathways. Life Sci. 13, 1623-1634.

Kuhar, M. J. & Murin, L. C. (1978) Sodium-dependant high affinity choline uptake. J. Neurochem. 30, 5-21.

Kuriyama, K., Weinstein, H. & Roberts, E. (1969) Uptake of GABA by mitochondrial and synaptosomal membrane fractions from mouse brain. Brain Res. 16, 479-492.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage, T_4. Nature 227, 680-685.

Lajtha, A. & Sershen, H. (1975) Inhibition of amino acid uptake by the absence of Na^+ in slices of brain. J. Neurochem. 24, 667-672.

Larsson, O. M., Krogsgaard-Larsen, P. & Schousboe, A. (1985) Characterization of the uptake of GABA, nipecotic acid and cis-4-OH-nipecotic acid in cultured neurones and astrocytes. Neurochem. Int. 7, 853-860.

Lees, G., Neuman, R., Beadle, D. J. & Benson, J. A. (1985) Flunitrazepam enhances GABA- and muscimol-induced responses in freshly dissociated locust neuronal somata. Pest. Sci. 16, 534.

Lehninger, A. L. (1975) Biochemistry, 2nd Editn., Worth Publishers, New York.

Levi, G. & Rateiri, M. (1973) GABA and glutamate uptake by subcellular fractions enriched in synaptosomes: critical evaluation of some methodological aspects. Brain Res. 57, 165-185.

Levi, G., Bertollini, A., Chen, J. & Rateiri, M. (1974) Regional differences in the synaptosomal uptake of [^{3}H] γ-aminobutyric acid and [^{14}C]glutamate and the possible role of exchange processes. J. Pharmacol. Exp. Therap. 188, 429-438.

Levi, G., Banay-Schwartz, M. & Rateiri, M. (1978) Studies on the release of endogenous and exogenous GABA and glutamate from rat brain synaptosomes. Neurochem. Res. 6, 275-285.

Levy, W. B., Redburn, D. A. & Cotman, C. W. (1973) Stimulus-coupled secretion of γ-aminobutyric acid from rat brain synaptosomes. Science 181, 676-678.

Lippa, A. S., Garrett, K. M., Tabakoff, B., Beer, B., Wennogle, L. P. & Meyerson, L. R. (1985) Heterogeneity of brain benzodiazepine receptors - effects of physiological conditions. Brain Res. Bull. 14, 189-195.

Lo, M. M. S. & Snyder, S. H. (1983) Two distinct solubilized benzodiazepine receptors: differential modulation by ions. J. Neurosci. 3, 2270-2279.

Loewi, O. (1921) Uber humorale Ubertragbarkeit der Herznervewirkung. Pflugers Arch. ges Physiol. 189, 239-242.

Loscher, W. (1980) Effect of inhibitors of GABA transaminase on the synthesis, binding, uptake and metabolism of GABA. J. Neurochem. 34, 1603-1608.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lummis, S. C. R. & Sattelle, D. B. (1985a) Insect central nervous system γ-aminobutyric acid. Neurosci. Lett. 60, 13-18.

Lummis, S. C. R. & Sattelle, D. B. (1985b) GABA and benzodiazepine binding sites in insect CNS. Pest. Sci. 16, 695-697.

Lummis, S. C. R. & Sattelle, D. B. (1986) Binding sites for [^{3}H]GABA, [^{3}H]flunitrazepam and [^{35}S]TBPS in insect CNS. Neurochem. Int. in press.

Lunt, G. G. (1985) Analysis of neurochemical data, in "Neurochemical Techniques in Insect Research" (Eds. Breer, H. & Miller, T. A.), Springer-Verlag, Berlin - Heidelberg - New York - Tokyo, 296-316.

Lunt, G. G. (1986) Insect neuronal GABA receptors: are they as sophisticated as their mammalian counterparts?. TINS. in press.

Lunt, G. G., Robinson, T. N., Miller. T., Knowles, W. P. & Olsen, R. W. (1985) The identification of GABA receptor binding sites in insect ganglia. Neurochem. Int. 7, 751-754.

Mackerer, C. R. & Kochman, R. L. (1978) Effects of cations and anions on the binding of [^{3}H]diazepam to rat brain. Proc. Soc. Exp. Biol. Med. 158, 393-397.

MacNeil, D. A., D'Amico, J. A., Horst, W. D., O'Brien, R. A. & Spirt, N. (1983) Influences of two atypical benzodiazepines, Ro5-3663 and Ro5-4864, on GABAergic mechanisms. Neurosci. Abstr. 9, 412.

Mann, E. & Enna, S. J. (1980) Phylogenetic distribuion of bicuculline-sensitive γ-aminobutyric acid (GABA) receptor binding. Brain Res. 184, 367-373.

Marangos, P. J., Patel, J., Boulenger, J-P. & Clark-Rosenberg, R. (1982) Characterization of peripheral type benzodiazepine binding sites in brain using [^{3}H]Ro5-4864. Mol. Pharmacol. 22, 26-32.

Marchbanks, R. M. (1967) The osmotically sensitive potassium and sodium compartments of synaptosomes. Biochem. J. 104, 148-157.

Marchbanks, R. M. & Israel, M. (1971) Aspects of acetylcholine metabolism in the electric organ of Torpedo marmorata. J. Neurochem. 18, 439-448.

Martin, D. L. & Smith III, A. A. (1972) Ions and the transport of GABA by synaptosomes. J. Neurochem. 19, 841-855.

Martin, I. L. & Candy, J. M. (1978) Facilitation of specific benzodiazepine binding in rat brain membrane fragments by a number of anions. Neuropharmacology 17, 993-998.

Martini, C., Rigacci, T. & Lucacchini, A. (1983) [^{3}H]Muscimol binding site on purified benzodiazepine receptor. J. Neurochem. 41, 1183-1185.

Matsuma, F. & Ghiasuddin, S. M. (1983) Evidence for similarities between cyclodiene type insecticides and

picrotoxin in their action mechanisms. J. Environ. Sci. Health B18, 1-14.

Matthews, G. (1983) Can we control insect pests? New Sci. 98, 369-372.

Maxwell, G. D., Tait, J. F. & Hildebrand, J. G. (1978) Regional synthesis of neurotransmitter candidates in the CNS of the moth, Manduca sexta. Comp. Biochem. Physiol. 61C, 109-119.

McAdoo, D. J. & Coggeshall, R. E. (1976) Gas chromatographic - mass spectrophotometric analysis of biogenic amines in identified neurones and tissues of Hirudo medicinalis. J. Neurochem. 26, 163-167.

McIlwain, H. & Bachelard, H. S. (1971) Biochemistry and the central nervous system. Churchill Livingstone, Edinburgh & London.

Meiners, B. M., Kehoe, P., Shaner, D. M., & Olsen, R. W. (1979) GABA receptor binding and uptake in membrane fractions of crayfish muscle. J.Neurochem. 32, 979-990.

Mellin, T. N., Busch, R. D. & Wang, C. C. (1983) Postsynaptic inhibition of invertebrate neuromuscular transmission by avermectin B_{1a}. Neuropharmacol. 22, 89-96.

Meyer, E. M., Engel, D. A. & Cooper, J. R. (1982) Acetylation and phosphorylation of choline following high or low affinity uptake by rat cortical synaptosomes. Neurochem. Res. 7, 749-759.

Meyer, E. P., Matute, C., Streit, P. & Nassel, D. R. (1986) Insect optic lobe neurones identifiable with monoclonal antibodies to GABA. Histochem. 84, 207-216.

Miller, T. A. (1980) Insect visceral and cardiac nerve-muscle systems, in "Insect Neurobiology and Pesticide Action (Neurotox '79)", London Society of Chemical Industry, 145-152.

Minnema, D. & Michaelson, I. A. (1985) A superfusion apparatus for the examinaton of neurotransmitter release from synaptosomes. J. Neurosci. Meth. 14, 193-206.

Mizuno, S., Ogawa, N. & Mori, A. (1982) Super high affinity binding site for [^{3}H]diazepam in the presence of Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+}. Neurochem. Res. 7, 1487-1493.

Mizuno, S., Ogawa, N. & Mori, A. (1983) Differential effects of some transition metal cations on the binding of β-carboline-3-carboxylate and diazepam. Neurochem. Res. 8, 873-880.

Mizuno, S., Ogawa, N. & Mori, A. (1984) Lack of specificity in cation effects on solubilized benzodiazepine receptor. Neurochem. Res. 9, 1729-1734.

Mohler, H. & Okada, T. (1977) Benzodiazepine receptor - demonstration in the central nervous system. Science 198, 849-851.

Mohler, H., Battersby, M. K. & Richards, J. G. (1980) Benzodiazepine receptor protein identified and visualized in brain tissue by photoaffinity labelling. Proc. Natl. Acad Sci. USA 77, 1666-1670.

Mohler, H., Sieghart, W., Richards, J. G. & Hunkeler, W. (1984) Photoaffinity labelling of benzodiazepine receptors with a partial inverse agonist. Eur. J. Pharmacol. 102, 191-192.

Mohler, H. Schoch, P., Richards, J. G., Haring, P., Takacs, B. & Stahli, C. (1986) Monoclonal antibodies: probes for structure and location of the GABA receptor/benzodiazepine receptor/ chloride channel complex, in "Benzodiazepine/GABA Receptors and Chloride Channels" (Eds. Olsen, R. W. & Ventner, J. C.), Alan R. Liss, 285-297.

Molinoff, P. B. & Kravitz, E. A. (1968) The metabolism

of gamma-aminobutyric acid (GABA) in the lobster nervous system - glutamic decarboxylase. J. Neurochem. 15, 391-409.

Morgan, I. G. (1976) Synaptosomes and cell separation. Neuroscience 1, 159-165.

Mulder, A. H., Yamamura, H. I., Kuhar, M. J. & Snyder, S. H. (1974) Release of acetylcholine from hippocampal slices by potassium depolarization: dependence on high affinity choline uptake. Brain Res. 70, 372-376.

Muller, M., Hogg, J. F. & De Duve, C. (1968) Distribution of tricarboxylic acid cycle enzymes and glyoxylate cycle enzymes between mitochondria and peroxisomes of Tetrahymena pyriformis. J. Biol. Chem. 243, 5385-5395.

Muller, W. E., Ickstadt, A. & Hopf, H. C. (1985) Peripheral benzodiazepine binding sites on striatal muscles of the rat - properties and effect of denervation. J. Neural Transmiss. 61, 35-42.

Muscatello, U. & Horne, R. W. (1968) Effect of the tonicity of some negative staining solutions on the elementary structure of membrane-bound systems. J. Ultrastruct. Res. 25, 73-83.

Nielsen, M., Braestrup, C. (1983) The molecular target size of brain TBPS binding sites. Eur. J. Pharmacol. 91, 321-322.

Nielsen, M., Braestrup, C. (1984) Target size of brain benzodiazepine receptor, GABA receptor and [^{35}S]-TBPS binding sites, in "Regulation of Transmitter Function" (Eds. Vici, E. S. & Magyar, K.), Akademia Kiado, Budapest, 429-432.

Nielsen, M., Braestrup, C. & Squires, R. F. (1978) Evidence for a late evolutionary appearance of brain-specific benzodiazepine receptors: an investigation of 18 vertebrate and 5 invertebrate species. Brain Res. 141, 342-346.

Olsen, R. W. (1981) GABA-benzodiazepine-barbiturate receptor interactions. J. Neurochem. 37, 1-13.

Olsen, R. W. (1982) Drug interactions at the GABA-receptor-ionophore complex. Ann. Rev. Pharmacol. Toxicol. 22, 245-277.

Olsen, R. W. & Snowman, A. M. (1985) Avermectin B_{1a} modulation of γ-aminobutyric acid/benzodiazepine receptor binding in mammalian brain. J. Neurochem. 44, 1074-1082.

Olsen, R. W., Lee, J. M. & Ban, M. (1975) Binding of γ-aminobutyric acid to crayfish muscle and its relationship to receptor sites. Mol. Pharm. 11, 566-577.

Olsen, R. W., Ticku, M. K. & Miller, T. A. (1978) Dihydropicrotoxinin binding to crayfish muscle sites possibly related to γ-aminobutyric acid receptor-ionophores. Mol. Pharmacol. 14, 381-390.

Olsen, R. W., Bergman, M. O., Van Ness, P. C., Lummis, S. C., Watkins, A. E., Napias, C. & Greenlee, D. V. (1981) GABA receptor binding in mammalian brain - heterogeneity of binding sites. Mol. Pharmacol. 19, 217-227.

Olsen, R. W., Fischer, J. B., King, R. G., Ransom, J. Y. & Stauber, G. B. (1984) Purification of the GABA/benzodiazepine/barbiturate receptor complex. Neuropharmacol. 23, 853-854.

Osborne, N. N., Briel, G. & Neuhoff, V. (1972) Distribution of GABA and other amino acids in different tissues of the gastropod mollusc, Helix pomatia, including in vitro experiments with[^{14}C]-glucose and [^{14}C]-glutamic acid. Intern. J. Neurosci. 1, 265-272.

Osborne, R. H., Bradford, H. F. & Jones, D. G. (1973) Patterns of amino acid release from nerve-endings

isolated from spinal cord and medulla. J. Neurochem. 21, 407-419.

Otsuka, M. E., Kravitz, E. A. & Potter, D. D. (1967) Physiological and chemical architecture of a lobster ganglion with particular reference to gamma-aminobutyrate and glutamate. J. Neurophysiol. 30, 725-752.

Paul, S. M., Skolnick, P. & Zatz, M. (1980) Avermectin Bla: an irreversible activator of the GABA-benzodiazepine-chloride ionophore receptor complex. Biochem. Biophys. Res. Commun. 96, 632-638.

Pichon, Y. (1974) The pharmacology of the insect nervous system, in "The Physiology of Insects" (Ed. Rockstein, M.), Academic Press, New York, 102-174.

Pieri, L., Polc, P., Bonetti, W., Burkard, W., Cumin, R., Scherschlicht, R. & Haefely, W. (1983) Some pharmacological effects of Ro5-4864, a specific ligand for the peripheral type of benzodiazepine binding sites. N. Sch's. Arch. Pharmacol. 322, (R95) 337.

Polc, P. & Schaffner, R. (1983) Electrophysiological effects of the peripheral-type benzodiazepine receptor ligand Ro5-4864 in cat spinal cord and rat hippocampus. Neurosci. Lett. Suppl. 14, 3288.

Pong, S. S. & Wang, C. C. (1982) Avermectin Bla modulation of 4-aminobutyric acid receptors in rat brain. J. Neurochem. 38, 375-379.

Pong, S. S., Wang, C. C. & Fritz, L. C. (1980) Studies on the mechanism of action of avermectin Bla: stimulation of release of 4-aminobutyric acid from brain synaptosomes. J. Neurochem. 34, 351-358.

Pong, S. S., DeHaven, R. & Wang, C. C. (1981) Stimulation of benzodiazepine binding to rat brain membranes and solubilized receptor complex by avermectin Bla and 4-aminobutyric acid. Biochim. Biophys. Acta 646, 143-150.

Pong, S. S., DeHaven, R. & Wang, C. C. (1982) A comparative study of avermectin Bla and other modulators of the 4-aminobutyric acid receptor chloride ion channel complex. J. Neurosci. 2, 966-971.

Potter, D. D. (1968) The chemistry of inhibition in crustaceans with special reference to gamma-aminobutyric acid, in "Structure and Fuction of Inhibitory Neuronal Mechanisms" (Eds. von Euler, C., Skoglund, S. & Soderberg, U.), Pergamon, Oxford, 359-370.

Ramon Y Cajal, S. (1888) Estructura de los centros nerviosos de las aves. Rev. Trimest. Histol. Norm. Patol. 1, 305-315.

Rapier, C. M., Harrison, R., Lunt, G. G. & Wonnacott, S. (1985) Neosurugatoxin blocks nicotinic acetylcholine receptors in the brain. Neurochem. Int. 7, 389-396.

Rastogi, S. K. & Ticku, M. K. (1985) A possible role of a GABAergic mechanism in the convulsant action of Ro5-4864. Pharmacol. Biochem. Behav. 23, 285-288.

Rateiri, M., Angelini, F. & Levi, G. (1974) A simple apparatus for studying the release of neurotransmitter from synaptosomes. Eur. J. Pharmacol. 25, 411-414.

Ray, J. W. (1964) The free amino acid pool of the cockroach (Periplaneta americana) central nervous system and the effects of insecticides. J. Insect Physiol. 10, 587-597.

Redburn, D., Biela, J., Shelton, D. & Cotman, C. (1975) Stimulus-secretion coupling in vitro: a rapid perfusion apparatus for monitoring efflux of transmitter substances from tissue samples. Anal. Biochem. 67, 268-278.

Regan, J. W., Yamamura, H. I., Yamada, S. & Roeske, W.

R. (1981) High affinity renal [^{3}H]flunitrazepam binding: characterization, localization and alteration in hypertension. Life Sci. 28, 991-998.

Roberts, E. (1964) Comparative aspects of the distribution of ninhydrin-reactive constituents in nervous tissue, in "Comparative Neurochemistry" (Ed. Richter, D.), Pergamon, Oxford, 401-408.

Roberts, E. & Frankel, S. (1950) γ-Aminobutyric acid in the brain; its formation from glutamic acid. J. Biol. Chem. 187, 55-63.

Robinson, T. N. & Olsen, R. W. (1987) GABA, in "Comparative Invertebrate Neurochemistry" (Eds. Lunt, G. G. & Olsen, R. W.), Croom-Helm, England, in press.

Rossier, J. (1985) The peptide 'explosion' and the new genetics, in "Neurotransmitters in Action" (Ed. Bousfield, D.), Elsevier Biomedical Press, Amsterdam - New York - Oxford, 143-150.

Sargent, P. B. (1977) Synthesis of acetylcholine by excitatory motor neurones of the leech. J. Neurochem. 40, 453-460.

Sattelle, D. B. (1980) Acetylcholine receptors of insects. Adv. Insect. Physiol. 15, 215-315.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660-672.

Schoch, P., Haring, P., Takacs, B., Stahli, C. & Mohler, H. (1984) A GABA/benzodiazepine receptor complex from bovine brain: purification, reconstitution, and immunological characterization. J. Recept. Res. 4, 189-200.

Schoch, P., Richards, J. G., Haring, P., Takacs, B., Stahli, C. Staehelin, T., Haefely, W. & Mohler, H. (1985) Co-localization of $GABA_A$ receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies. Nature 314, 168-171.

Schoemaker, H., Bliss, M. & Yamamura, H. I. (1981) Specific high-affinity saturable binding of [^{3}H]Ro5-4864 to benzodiazepine binding sites in rat cerebral cortex. Eur. J. Pharmacol. 71, 173-175.

Schon, F. E. & Kelly, J. S. (1974) The characterization of [^{3}H]GABA uptake into the satellite glial cells of rat sensory ganglia. Brain Res. 66, 289-300.

Schousboe. A., Wu, J-Y. & Roberts, E. (1973) Purification and characterization of

4-aminobutyrate-2-ketoglutarate transaminase from the mouse brain. Biochemistry 12, 2868-2873.

Schousboe, A., Hjeds, H., Engler, J., Krogsgaard-Larsen, P. & Wood, J. D. (1986) Tissue distribution, metabolism, anticonvulsant efficacy and effect on brain amino acid levels of the glial-selective γ-aminobutyric acid transport inhibitor 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol in mice and chicks. J. Neurochem. 47, 758-763.

Sellstrom, A., Venema, R. & Henn, F. (1976) Functional assessment of GABA uptake or exchange by synaptosomal fractions. Nature 264, 652-653.

Shepherd, D. & Tyrer, N. M. (1985) Inhibition of GABA uptake potentiates the effects of exogenous GABA on locust skeletal muscle. Comp. Biochem. Physiol. 82C, 315-321.

Sherrington, C. S. (1925) Remarks on some aspects of reflex inhibition. Proc. Roy. Soc. (Edinburgh) B. 97, 519-545.

Sieghart, W. & Drexler, G. (1983) Irreversible binding of [^{3}H]flunitrazepam to different proteins in various brain regions. J. Neurochem. 41, 47-55.

Sieghart, W. & Karobath, M. (1980) Molecular heterogeneity of benzodiazepine receptors. Nature 286, 285-287.

Sieghart, W., Mayer, A. & Drexler, G. (1983) Properties of [^{3}H]flunitrazepam binding to different benzodiazepine binding proteins. Eur. J. Pharmacol. 88, 291-299.

Sigel, E. & Barnard, E. A. (1984) A GABA/benzodiazepine receptor complex from bovine cerebral cortex. J. Biol. Chem. 259, 7219-7223.

Sigel, E., Mamalaki, C. & Barnard, E. A. (1985) Reconstitution of the γ-aminobutyric acid-benzodiazepine receptor complex from bovine cerebral cortex into phospholipid vesicles. Neurosci. Lett. 61, 165.

Sigel, E., Stevenson, F. A., Mamalaki, C. & Barnard, E. A. (1983) GABA/benzodiazepine receptor complex of bovine cerebral cortex - purification and partial characterization. J.Biol.Chem.258, 6965-6971.

Simmonds, M. A. (1983) Multiple GABA receptors and associated regulatory sites. TINS. 6, 279-281.

Simmonds, M. A. (1985) Antagonism of flurazepam and other effects of Rol5-1788, PK8165 and Ro5-4864 on the $GABA_A$ receptor complex of rat cuneate nucleus. Eur. J.

Pharmacol. 117, 51-60.

Simon, J. R. & Kuhar, M. J. (1976) High affinity choline uptake: ionic and energy requirements. J. Neurochem. 27, 93-99.

Smart, T. G., Constanti, A., Bilbe, G., Brown, D. A. & Barnard, E. A. (1983) Synthesis of functional chick brain GABA-benzodiazepine-barbiturate receptor complexes in mRNA-injected Xenopus oocytes. Neurosci. Lett. 40, 55-59

Smith, A. E. (1973) A study of the variation with pH of the solubility and stability of some metal ions at low concentrations in aqueous solution, part I. Analyst 98, 65-68.

Snodgrass, S. R. & Iversen, L. L. (1973) Effects of aminooxyacetic acid on [^{3}H]GABA uptake by rat brain slices. J. Neurochem. 20, 431-439.

Snodgrass, S. R., Hedley-Whyte, E. T. & Lorenzo, A. V. (1973) GABA transport by nerve ending fractions of cat brain. J. Neurochem. 20, 771-782.

Stapleton, A. G. (1986) Studies on L-glutamic acid decarboxylase from locust brain. PhD thesis, UMIST, England.

Stephenson, F. A., Casalotti, S. O., Mamalaki, C. & Barnard, E. A. (1986) Antibodies recognizing the $GABA_A$/benzodiazepine receptor including its regulatory sites. J. Neurochem. 46, 854-861.

Sweetnam, P. M. & Tallman, J. F. (1986) Regional differences in brain benzodiazepine receptor carbohydrates. Mol. Pharmacol. 29, 299-306.

Su, Y. Y. T., Wu, J-Y. & Lam, D. M. K. (1979) Purification of L-glutamic acid decarboxylase from catfish brain. J. Neurochem. 33, 169-179.

Su, Y. Y. T., Wu, J-Y. & Lam, D. M. K. (1983) Species specificities of L-glutamic decarboxylase: immunochemical comparisons. Neurochem. Int. 5, 587-592.

Sugden, P. H. & Newsholme, E. A. (1977) Activities of ChAT, AChE, GAD, GABA-T and carnitine acetyl transferase in nervous tissue from some vertebrates and invertebrates. Comp. Biochem. Physiol. 56C, 89-94.

Supavilai, P. & Karobath, M. (1980) Effect of temperature and chloride ions on the stimulation of [^{3}H]flunitrazepam binding by the muscimol analogues, THIP and piperidine-4-sulphonic acid. Neurosci. Lett. 19, 337-341.

Supavilai, P. & Karobath, M. (1981) In vitro modulation by avermectin Bla of the GABA/benzodiazepine receptor complex of rat cerebellum. J. Neurochem. 36, 798-803.

Susz, J. P., Haber, B. & Roberts, E. (1966) Purification and some properties of mouse brain L-glutamic acid decarboxylase. Biochemistry 5, 2870-2877.

Szamraj, O. I., Miller, T. A. & Olsen, R. W. (1986) Cage convulsant [^{35}S]TBPS binding to GABA receptor-chloride channel complex in invertebrate tissues. Abstr. Soc. Neurosci. in press.

Tallan, H. H. (1962) A survey of amino acids and related compounds, in "Nervous Tissue, Amino Acid Pool" (Ed. Holden, J. T.), Elsevier, Amsterdam and London.

Tallman, J. F. & Gallager, D. W. (1985) The GABA-ergic system: a locus of benzodiazepine action. Ann. Rev. Neurosci. 8, 21-44.

Tallman, J. F., Thomas, J. W. & Gallagher, D. W. (1978) GABAergic modulation of benzodiazepine binding site sensitivity. Nature, 274, 383-385.

Tanaka, K. & Matsumura, F. (1985) Action of avermectin B_{1a} on the leg muscles and the nervous system of the American cockroach. Pest. Biochem. Physiol. 24, 124-135.

Tanaka, K., Scott, J. G. & Matsumura, F. (1984) Picrotoxin receptor in the central nervous system of the American cockroach: its role in the action of cyclodiene insecticides. Pest. Biochem. Physiol. 22, 117-127.

Telford, J. N. & Matsumura, F. (1970) Dieldrin binding in subcellular nerve components of cockroaches. An electron microscopic and autoradiographic study. J. Econ. Entom. 63, 795-800.

Thomas, J. W. & Tallman, J. F. (1981) Characterization of photoaffinity labelling of benzodiazepine binding sites. J. Biol. Chem. 256, 9838-9842.

Ticku, M. K. & Ramanjaneyulu, R. (1984) Ro5-4864 inhibits the binding of [^{35}S]TBPS to rat brain membranes. Life Sci. 34, 631-638.

Tsukada, Y., Uemura, K., Hirano, S. & Nagata, Y. (1964) Distribution of amino acids in the brain of different species, in "Comparative Neurochemistry" (Ed. Richter, D.), Pergamon, Oxford, 179-183.

Udenfriend, S. (1950) Identification of -aminobutyric acid in brain by the isotope derivative method. J. Biol. Chem. 187, 33-59.

Usherwood, P. N. R.(1978) Amino acids as transmitters.

Adv. Comp. Phys. Biochem. 7, 227-309.

Usherwood, P. N. R. & Cull-Candy, S. G. (1975) Pharmacology of somatic nerve-muscle synapses, in "Insect Muscle" (Ed. Usherwood, P. N. R.), Academic Press, London, New York and San Francisco, 207-280.

Usherwood, P. N. R. & Grundfest, H. (1964) Inhibitory postsynaptic potentials in grasshoper muscle. Science 143, 817-818.

Usherwood, P. N. R. & Grundfest, H. (1965) Peripheral inhibition in skeletal muscle of insects. J. Neurophysiol. 28, 497-518.

Van Marle, J., Piek, T., Lammerste, Th., Lind, A. & Van Weeren-Kramer, J. (1985) Selectivity of the uptake of glutamate and GABA into two morphologically distinct insect neuromuscular synapses. Brain Res. 348, 107-111.

Villiger, J. W. (1985) Characterization of peripheral-type benzodiazepine recognition sites in the rat spinal cord. Neuropharmacology 24, 95-98.

Wang, Y. -J., Salvaterra, P. & Roberts, E. (1979) Characterization of [^{3}H]muscimol binding to mouse brain membranes. Biochem. Pharmacol. 28, 1123-1128.

Wastek, G. J., Speth, R. C., Reisine, T. D. & Yamamura,

H. I. (1978) The effect of gamma-aminobutyric acid on [^{3}H]flunitrazepam binding in the rat brain. Eur. J. Pharmacol. 50, 445-447.

Watts, S. D. M. & Atkins, A. M. (1984) Kinetics of 4-amino-2-oxoglutarate aminotransferase from Nippostrongylus brasiliensis. Mol. Biochem. Parasit. 12, 207-216.

Weissman, B. A., Bolger, G. T., Isaac, L. Paul, S. M. & Skolnick, P. (1984a) Characterization of the binding of [^{3}H]Ro5-4864, a convulsant benzodiazepine, to guinea pig brain. J. Neurochem. 42, 969-980.

Weissman, B. A., Cott, J., Hommer, D., Paul, S. M. & Skolnick, P. (1984b) Electrophysiological and pharmacological actions of the convulsant benzodiazepine, Ro5-4864. Eur, J, Pharmacol. 97, 257-263.

Werman, R. (1966) A review - criteria for the identification of a central nervous system transmitter. Comp. Biochem. Physiol. 18, 745-766.

Wheeler, D. D. (1978) Some problems inherent in transport studies in synaptosomes. J. Neurochem. 30, 109-120.

Whittaker, V. P. (1973) The biochemistry of synaptic transmission. Naturewissenschaften 60, 281-289.

Whittaker, V. P. (1984) The synaptosome, in "Handbook of Neurochemistry", 2nd Editn. (Ed. Lajtha, A.), Plennum Press, New York, 1-35.

Whitton, P. S., Nicholson, R. A. & Strang, R. H. C. (1986) Metabolism of taurine by synaptosomal preparations from insects. Biochem. Soc. Trans. 14, 609-610.

Williams, L. T. & Lefkowitz, R. J. (1978) Receptor binding studies in adrenergic pharmacology, Raven Press, New York, 27-41.

Williams, M. & Risley, E. A. (1979) Characterization of the binding of [^{3}H]muscimol, a potent -aminobutyric acid agonist, to rat brain synaptosomal membranes using a filtration assay. J. Neurochem. 32, 713-718.

Williams, M. & Risley, E. A. (1982) Interaction of avermectins with [^{3}H] -carboline-3-carboxylate ethyl ester and [^{3}H]diazepam binding sites in rat brain cortical membranes. Eur. J. Pharmacol. 77, 307-312.

Williams, M. & Risley, E. A. (1984) Avermectin interactions with benzodiazepine receptors in rat cortex

and cerebellum *in vitro*. J. Neurochem. *42*, 745-753.

Williams, M. & Yarborough, G. G. (1979) Enhancement of *in vitro* binding and some of the pharmacological properties of diazepam by a novel antihelmintic agent, avermectin Bla. Eur. J. Pharmacol. *56*, 273-276.

Witte, P. U. & Matthaei, H. (1980) Mikrochemische methoden fur neurobiologische unterschungen. Springer, Berlin, Heidelberg, New York, 99-101.

Wonnacott, S. & Marchbanks, R. M. (1976) Inhibition by botulinum toxin of depolarization-evoked release of [^{14}C]acetycholine from synaptosomes *in vitro*. Biochem. J. *156*, 701-712.

Wood, J. D. & Sidhu, H. S. (1986) Uptake of γ-aminobutyric acid by brain tissue preparations - a reevaluation. J. Neurochem. *46*, 739-744.

Wright, D. J. (1986) Biological activity and mode of action of avermectins, in "Neuropharmacology and Pesticide Action" (Eds. Ford, M. G., Lunt, G. G., Reay, R. C. & Usherwood, P. N. R.), Ellis & Horwood, England, 174-202.

Wu, R. & Racker, E. (1959) Regulatory mechanisms in carbohydrate metabolism. J. Biol. Chem. *234*, 1029-1035.

Wu, J-Y., Matsuda, T. & Roberts, E. (1973) Purification and characterization of glutamate decarboxylase from mouse brain. J. Biol. Chem. 248, 3029-3034.

Wu, J-Y., Chude, O., Weber, B., Driskell, J. & Roberts, E. (1976) Properties of L-glutamate decarboxylase from crayfish. Trans. Am. Soc. Neurosci. 7, 190.

Yamamura, H. I. & Snyder, S. H. (1972) Synaptosome uptake of choline. Two kinetically distinct processes. Science 178, 626-628.

Yamamura, H. I. & Snyder, S. H. (1973) High affinity transport of choline into synaptosomes of rat brain. J. Neurochem. 21, 1355-1374.

Zhu, J. Q., Sun, Y. & Wang, X. G. (1986) Selected inhibition of aminooxyacetic acid on high affinity gamma-aminobutyric acid uptake system in mice brain homogenate. Acta. Pharmacol. Sin. 7, 13-15.

Zukin, S. R., Young, A. B. & Snyder, S. H. (1974) Gamma-aminobutyric acid binding to receptor sites in rat central nervous system. Proc. Natl. Acad. Sci. USA 71, 4802-4807.